TISSUE CULTURE AND ELECTROPHORETIC STUDIES OF PINEAPPLE (*Ananas comosus*) AND RELATED SPECIES

By

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This dissertation is dedicated to my husband, Steve, for his constant support, understanding, love, and help through these last years while we were students and parents.

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KEY TO ABBREVIATIONS

ATP:	adenosine 5' triphosphate disodium salt
BA:	6-benzylaminopurine (N^6 benzyladenine)
CW:	coconut water
H:	histidine buffer
IAA:	indole-3-acetic acid
IBA:	indole-3-butyric acid
K:	K buffer
KIN:	kinetin
LBTC:	lithium borate/tris citrate buffer
MS:	Murashige and Skoog basal medium formulation
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide
NAA:	2-naphthaleneacetic acid
NAD:	beta-nicotinamide adenine dinucleotide
NADP:	beta-nicotinamide adenine dinucleotide phosphate
Na_2EDTA	disodium ethylenediaminetetracetic acid
NBT:	nitro blue tetrazolium
PMS:	phenazine methosulfate
R_b:	band migration relative to band sample
R_f:	band migration relative to borate front

TB:	tris borate buffer
TC:	tris citrate buffer
Tris:	tris-(hydroxymethyl) amino methane
2,4-D:	(2,4-dichlorophenoxy)acetic acid

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Tissue culture studies were conducted with pineapple (*Ananas comosus*) and *A. bracteatus* var. *tricolor* in order to evaluate the feasibility of in vitro techniques as a method for the rapid multiplication of desirable genotypes. A system utilizing shake cultures derived from axillary buds of crowns, slips, and stems was devised. This system resulted in the efficient production of large numbers of pineapple plantlets that were easily established in soil under greenhouse conditions. The regenerated plants were characterized morphologically and on the basis of isozyme banding patterns. High levels of phenotypic variability, especially for leaf morphology, were observed shortly after the small plants were transferred to the soil. After one

year only 4 variant phenotypes persisted in the 2000 regenerated plants. No isozyme variants were identified.

Electrophoretic characterization of Ananas with emphasis on economically important pineapple cultivars was also conducted to help establish phenetic relationships. A method was developed whereby leaf sap could be quickly extracted and analyzed for isozyme polymorphisms using starch gel electrophoresis. One hundred and ninety combinations consisting of 38 enzyme activity stains and 5 electrophoretic buffer systems were evaluated for isozyme banding resolution. From this survey, 8 enzyme staining systems giving good banding resolution were identified. Three of these, peroxidase, phosphoglucomutase, and malate dehydrogenase, were polymorphic. Two segregating seedling populations were utilized to establish a genetic model for the 3 polymorphic enzymes. The genetic analysis revealed that both peroxidase and phosphoglucomutase were controlled by 3 independent, monomeric loci.

A taxonomic survey of 67 Ananas genotypes was conducted utilizing the peroxidase and phosphoglucomutase staining systems. Fifteen of the 27 pineapple cultivars and 5 of the 7 species analyzed could be uniquely distinguished using the 2 enzyme systems. Ananas comosus, one of the species that could not be unambiguously distinguished, was highly polymorphic due to the large number of cultivars included in the species. Ananas monstrosus, the other species without a unique isozyme phenotype, was confirmed to be a simple crown

mutation of A. comosus that occurs in many cultivars and therefore is not considered a valid species. Most of the 33 feral types analyzed could be included under species with similar banding patterns. Phenetic relationships of the genotypes based on cluster analysis dendograms were established.

CHAPTER I
INTRODUCTION

Pineapple is one of the most important fruit crops in the world. Nearly 12 million metric tons are produced annually in the tropical and subtropical regions of the world (FAO Production Yearbook, 1984). It is particularly well suited to arid and semiarid tropical locations where few other crops do well.

One reason for the popularity of pineapple is that it has proved very amenable to large-scale commercial production and has good canning qualities. Most of the world production can be attributed to 'Smooth Cayenne' and its sports. 'Smooth Cayenne' is characterized by its relatively smooth leaves (spiny tip) and large cylindrical fruit with good canning qualities. However, it is susceptible to several economically important diseases and does not produce slips, which are needed for propagation. Numerous high quality spiny cultivars also exist that are more adapted to tropical regions, where 'Smooth Cayenne' does not develop good fruit qualities, or are better suited for the fresh market. The presence of spines and the scarcity of planting material on these cultivars severely restricts their

suitability to large scale planting. Pineapple breeding efforts have been directed mainly at the improvement of 'Smooth Cayenne', because most of the breeding programs have been conducted by large commercial companies whose production is dependent on the specific canning characteristics of this cultivar.

The genus Ananas, to which pineapple belongs, is poorly characterized taxonomically. One explanation for this may be that the center of origin and diversity of this genus is located in the isolated regions of the upper Amazonian River basin. This lack of characterization limits the ability of plant breeders to utilize the variability present in both A. comosus and related species.

The objectives of this research were 1) to develop an efficient in vitro propagation system for pineapple; 2) to morphologically and electrophoretically evaluate the genetic fidelity of the regenerated plants; 3) to identify techniques that are useful for visualizing isozymes in Ananas; 4) to survey the available Ananas germplasm to determine if variability in isozyme banding patterns exists; 5) to study the possible genetic basis for variability in isozyme banding patterns; 6) to uniquely identify Ananas species, including pineapple cultivars, and to categorize feral types into species using isozyme polymorphism; and 7) to establish phenetic relationships among Ananas genotypes based on isozyme banding patterns.

CHAPTER II
LITERATURE REVIEW

Ananas Taxonomy

Pineapples [Ananas comosus (L.) Merr.] have been studied and characterized by naturalists and botanists for hundreds of years; however, there has been much variation in their taxonomic treatment. Some botanists believe Ananas is a monotypic genus, i.e., a single species, comprised of many varieties. Others have considered the genus to include many species, which are often separated on the basis of a few, slight character variations (Collins, 1960).

The latest classification of the genus Ananas was by Smith and Downs (1979). Smith was the botanist who also classified most of the Bromeliaceae (the Ananas family). The species considered valid in this latest treatment are A. ananassoides, A. bracteatus, A. comosus, A. fritzmuelleri, A. lucidus, A. nanus, and A. parguazensis. Classification is based upon the presence or absence of a crown (coma foliaceous), syncarp size, presence and direction of spines, and floral bract characteristics. This is the most comprehensive survey of the pineapple genus to date, providing botanists for the first time with a taxonomic key.

A chronological review of pineapple taxonomy was later made by Antoni (1983), indicating that the present classification of the genus Ananas is highly confused and questioning the validity of some species, e.g., A. monstrosus. The same author stressed the importance of using techniques such as tissue culture and electrophoretic separation of isozymes to differentiate species.

Pineapple Propagation

Pineapple is a self-incompatible perennial crop that is vegetatively propagated. The crop is usually cultivated in high density fields. The spineless cultivar 'Smooth Cayenne' is planted in Hawaii at 60,000 to 80,000 plants per ha. (Py and Tisseau, 1965). In other regions where spiny cultivars are used, only 15,000 to 35,000 plants are planted per ha. (Py and Tisseau, 1965). At such high planting densities, propagules are a major limiting factor in the establishment of new fields. The problem can be even more extreme in the case of newly developed cultivars. The number of propagules per pineapple plant varies with the cultivar. However, a typical plant produces between 6-10 propagules for crop cycle (18-22 months). These propagules include the crown (produced on the fruit apex), slips (produced on the fruit peduncle), and suckers (produced on the plant stem). A solitary sucker is usually left in place to produce the subsequent ratoon fruit. Culturally, slips are the most desired propagule. They are more favorable than crowns

because they are uniform, root faster, and can be planted deeper, giving rise to a more uniform stock of plants.

The most economically important cultivar, 'Smooth Cayenne', has low slip production. Slip production is further reduced in this cultivar when it is planted at high densities to produce a fruit size acceptable for efficient processing (Ravcof and Yamane, 1970). Crowns are sold with the fruit in fresh market situations, thereby further reducing the amount of propagules.

Several methods have been developed over the past 40 years to increase the amount of propagules (Py, 1979). One method, developed by Macluskie (1939), is to section the mature stems. The individual pieces are placed in sand boxes and incubated in a warm moist place. After several weeks the axillary buds grow and form adventitious roots. This technique was later tried by several researchers using sections of crowns and slips (Tkatchenko, 1947; Collins, 1960; Py and Estanove, 1964). A second method consists of propagating a single leaf with intact axillary bud dissected from the crown or slip (Seow and Wee, 1970). This method is laborious and produces a limited amount of plantlets per crown.

The most recent advance in pineapple multiplication employs plant morphactins (Sanford and Abdul, 1971). Plant morphactins are a diverse group of synthetic chemicals which dramatically alter the morphological development of the plant. Morphactins in pineapple are used in combination with

flower-inducing ethylene compounds, e.g., ethrel. Mature plants (12-16 month old) are first induced to flower. The flowering process is then converted into a multiplicant process by the application of a morphactin, e.g., Maintain CF-125. Using this method, up to 12 "sliplets" per plant can be produced (Ravcof and Yamane, 1970). This is an economical and rapid (2 years) method that allows the production of over half a million vigorous propagules similar to slips per ha. Planting material for most large-scale commercial plantings of 'Smooth Cayenne' types is now produced by this method of multiplication (Krezdorn, personal communication, 1985).

All of these methods require large fields to establish plants. Rapid and more efficient pineapple propagation systems still limit the establishment of new commercial plantings. Tissue culture appears to be a very attractive alternative method of multiplication and has particular utility in situations where clonal material is scarce.

Pineapple Tissue Culture

Pineapples have been regenerated in vitro from the culture of shoot tips, axillary buds, and immature syncarps. Direct plant production from excised axillary buds was first reported by Aghion and Beauchesne (1960). A single plantlet was obtained from each cultured axillary bud on solid, half strength Knop's medium supplemented with 15% (v/v) coconut water (CW). Sita et al. (1974) also regenerated single

plants from individual slip shoot tips on a solid medium of Knudson major salts and Nitsch minor salts supplemented with 5.4 uM NAA.

Mapes (1974) was the first to describe a method for multiple shoot production directly from axillary buds present in cultured shoot. The shoot tips were placed in liquid Murashige and Skoog (MS) medium supplemented with 0.22 mM adenine to initiate proliferation. Subsequently, they were transferred to solid MS medium supplemented with 0.15 mM adenine to allow shoot development. The proliferating mass of shoots was termed a protocorm-like structure. Similar results were obtained by Teo (1974) and Pannetier and Lanaud (1976). Wakasa et al. (1978) reported the initiation of shoots from protocorm-like calli derived from immature syncarps, axillary buds of suckers and slips, immature crowns, and immature slips cultured on solid MS media containing 5.4-54.0 mM NAA and 4.4-44.0 uM BA. The best results were obtained on medium supplemented with 10.8 uM NAA and 8.8 uM BA. Wakasa et al. (1978) described 2 types of protocorm-like calli. Calli derived from axillary buds were described as nodule-like in appearance, while the calli derived from axillary buds were described as globule-like.

Mathews et al. (1976) demostrated that liquid culture medium appeared to be most suitable for shoot proliferation from axillary bud cultures. They used an MS medium containing 9.7 uM NAA, 9.8 uM IBA, and 9.2 uM KIN. Shoots were initiated on solid medium supplemented with 4.4 uM BA

and 4.9 uM IBA (Mathews and Rangan, 1979). Later studies by these authors indicated that callus could also be initiated from axillary bud cultures on MS medium supplemented with 54.0 uM NAA, 15% (v/v) CW, and 0.04% (w/v) casein hydrolysate. Shoot proliferation occurred on MS medium supplemented with 9.7 uM NAA, 9.8 uM IBA, and 9.2 uM KIN. Root formation occurred on White's medium containing 0.27 uM NAA and 0.196 uM IBA. Zepeda and Sagawa (1981) reported limited shoot proliferation from axillary buds cultured in liquid MS medium supplemented with 25% (v/v) CW.

Shoot proliferation from hybrid embryo-derived callus was reported by Srinivasa Rao et al. (1981) on MS medium with 10.8 uM NAA, 9.8 uM IBA, and 11.0 uM BA. Immature leaf explants have also been used for shoot proliferation on MS solid medium containing 9.7 uM NAA, 9.8 uM IBA, and 48.4 uM BA (Mathews and Rangan, 1979).

Drew (1980) published a report on pineapple tissue culture theorizing the possibility of obtaining 100,000 plants from a single axillary bud in 12 months. There is no mention in the literature in regard to the number of regenerated plants established in soil, with the exception of Wakasa et al. (1978) who reported growing 400 regenerated plants.

Somaclonal Variation

The term somaclonal variation was coined by Larkin and Scowcroft (1981) to describe the phenotypic variation

observed in plants regenerated from somatic tissue in vitro. Somaclonal variation appears to be common to all in vitro propagation systems. However, the quantity and quality of the variation differs greatly with the genotype and propagation protocol.

A number of reviews have been written on this subject (Bayliss, 1980; Larkin and Scowcroft, 1981; Orton, 1983a; 1983b; 1984; Evans et al. 1984). Agromonically useful somaclonal variation has been reported in many crop species, e.g., alfalfa (Johnson et al., 1980), maize (Edallo et al., 1981), oats (McCoy et al., 1982), potato (Shepard et al., 1980), sugarcane (Heinz and Mee, 1969), tobacco (Chaleff and Keil, 1981), tomato (Evans et al., 1984), and wheat (Larkin et al., 1984).

The variation observed in tissue culture-produced somaclones may be stable and heritable or it may be transient, nonheritable, and epigenetic in nature. It is particularly critical for the scientist to distinguish between types of phenotypic variation. Epigenetic variation, because it is nonheritable, has limited usefulness to cultivar improvement.

A wide range of chromosomal alterations are possible in tissue culture-regenerated plants. Single base pair alterations or deletions (Edallo et al., 1981) represent one end of the spectrum, while major karyotypic alterations, including the doubling or deletion of complete sets of

chromosomes (Janick et al., 1977), represent the other extreme.

Stable, heritable variation implies genetic variability. This genetic variability may be due to pre-existing heterogeneity present in the cells of the explant and/or de novo genetic alterations occurring during the culturing process (Evans et al., 1984). Unfortunately, scientists have not been able to determine the relative amount of variation attributable to these 2 sources (Larkin and Scowcroft, 1981).

The full range of genetic variability that can be generated and expressed in tissue culture-regenerated plants has not yet been established. It does appear, however, that the full range of genetic variability present in cultured cells is not present in the regenerated plants (Edallo et al., 1981). The regeneration process may be serving as a selective screen for those cells that are genetically competent to form plants.

Perhaps the best documented source of variation is the explant. Barbier and Dulieu (1980) observed higher levels of somaclonal variation in tobacco plants that were regenerated from protoplasts and newly initiated callus cultures in comparison with plants regenerated from well established callus cultures. Wakasa (1979) also reported significant differences in the amount of somaclonal variation observed for vegetative morphology in 'Smooth Cayenne' pineapple plants regenerated from 3 explant sources. He found that

plants regenerated from syncarp (immature fruit) tissues exhibited 100% variability, while plants derived from axillary buds of crowns and slips were 34% variable. The lowest levels of variation (7%) were observed in plants regenerated from small crowns. Varibilities in leaf wax, color, spines, and foliage density were observed in the plants derived from the syncarp, while only spine variability was observed in the plants from slips and axillary buds. It should be noted that this study was done on up to 2-year-old regenerated plants that were grown in the greenhouse, and no long-term study on the stability of this variation was conducted.

The amount of time in culture can also influence the variability of regenerated plants. Karyotypic changes have been correlated with increasing time in culture (Bayliss, 1980). Increasing time in culture has been shown to increase somaclonal variation in tobacco (Syono and Furuya, 1972), Chrysanthemum (Bush et al., 1976; Sutter and Langhans, 1981), and oats (McCoy, 1980 cited by Reisch, 1983). In contrast, long-term cultures of corn were reported to be stable (McCoy, 1980 cited by Reisch, 1983).

The phytohormones used in the culture medium are another possible factor affecting somaclonal variation. High concentrations (18 uM) of the auxin 2,4-D were reported to produce unique variation in regenerated barley plants that was not observed in plants regenerated using lower concentrations (Deambrogio and Dale, 1980). Similarly, a

medium supplemented with NAA (27 uM) and KIN (0.5 uM) was shown to produce significantly more karyotypic alterations in regenerated Haworthia plants than a medium supplemented with 0.6 uM IAA (Ogihara, 1981).

The genotype has also been shown to influence somaclonal variation. Liu and Chen (1976) evaluated 8 sugarcane cultivars and found that regenerated plants from the cultivars ranged from 1.8% variable to 34% variable. Skirvin and Janick (1976) found somaclonal variation cultivar differencies in Pelargonium sp. DeWald and Moore (1987) indicated in their review that certain crops, e.g., polyploids and asexually propagated crops in which sexual fertility is not important, appear to tolerate higher levels of chromosomal variation produced via somaclonal variation than other crops.

Applications of Isozyme Analysis in Horticultural Plant Systematics

Isozymes are multiple molecular forms of an enzyme having the same substrate specificity (Markert and Moller, 1959). Hunter and Markert (1957), using the starch gel electrophoresis technique developed by Smithies (1955), demonstrated that isozymes can be visualized on starch gels stained with specific histochemical stains. Bands appearing on a starch gel correspond to different alleles and/or loci coding for a specific enzyme.

Isozymes have been used extensively as genetic markers in plants. They have a number of advantages over conventional markers. Isozymes are generally not affected by the morphological or physiological status of the plant. They interact codominantly at a single locus, rarely exhibit epistatic interactions, and can be detected in a variety of tissues (Tanksley and Rick, 1980; Tanksley et al., 1982; Tanksley, 1983).

The use of electrophoretic analysis to characterize isozyme variation as an aid for plant systematics is now widely applied and has been the subject of several reviews (Gottlieb, 1977; Crawford, 1983). The electrophoretic phenotype can be directly equatable with genotype, unlike many morphological characters, which may demonstrate a great deal of enviromental or developmental plasticity (Crawford, 1983; Gottlieb, 1977).

In plant systematics, isozyme electrophoresis has been successfully utilized for both cultivar and species identification by providing a way to quantitatively assess the genetic variation among conspecific populations and the genetic divergence among congeneric species. Other important applications include the resolution of polyploid taxa and insight into their origins, the investigation of the origin of cultivated crops, and the testing of broad systematic or evolutionary hypotheses.

Reliable identification of cultivars using classical methods based on morphological and physiological characters

has become increasily difficult, partially due to the large number of lines or varieties now available. Isozymes as bio-chemical markers for cultivar identification have proven reliable, consistent, and essentially unaffected by enviromental conditions (Bailey, 1983). They can be used as "fingerprints" to aid in the visual identification of cultivars (Pierce and Brewbaker, 1973). The utility of this technique has already been demonstrated in a number of species, e.g., Anthurium (Kobayashi et al., 1987), alfalfa (Quiros, 1980), apple (Menendez et al., 1986; Weeden and Lamb, 1985), avocado (Torres et al., 1978a), barley (Bassiri, 1976), bean (Bassiri and Rouhani, 1977; Weeden, 1984), Camellia (Wendel and Parks, 1983), Citrus (Torres et al., 1978b), grape (Schwennesen et al., 1982), grasses (Wilkinson and Beard, 1972), maize (Cardy and Kannenberg, 1982), Musa (Jarret and Litz, 1986b), peach (Carter and Brock, 1980), pear (Santamour and Demuth, 1980), pecan (Mielke and Wolfe, 1982), poinsettia (Werner and Sink, 1977), rice (Sarkar and Bose, 1984), strawberry (Bringhurst et al., 1981), and wheat (Menke et al., 1973).

The use of isozymes in the investigation of plant taxonomy, particularly species circumscription is relatively recent. Zymogram techniques have been used most extensively in Nicotiana. Characteristic enzyme patterns for most Nicotiana species have been observed using proteins (Hart and Bathia, 1967), peroxidase alone (Sheen, 1970), and in combination with esterases (Smith et al., 1970). Species

identifications using isozymes have also been reported in Datura (Conklin and Smith, 1971), Citrus (Button et al., 1976), Musa (Jarret and Litz, 1986c), and Pistacia (Loukas and Pontikis, 1979).

Specific isozymes or proteins have been used to identify particular genomes in polyploids. In bananas, Jarret and Litz (1986a) confirmed the bispecific origin of the triploid 'Chato' from Musa acuminata and Musa balbisiana using leaf isozyme polymorphisms. An allopolyploid origin for the apple has been proposed based on inheritance data of 7 pollen enzymes (Chevreau et al., 1985). Isozyme analysis of Gossypium hirsutum, a natural allopolyploid, has shown that esterases exhibit greater intergenomic variability than intraspecific or intravarietal variability (Cherry et al., 1970). In a later study, Cherry et al. (1971) showed that evolutionary changes had occurred in the species that were reflected in isozyme differences.

One of the most common uses of isozymes has been to assess the validity of proposed parentages of cultivars and progeny. This has been done in apple (Chyi and Weeden, 1984; Weeden and Lamb, 1985), avocado (Golching et al., 1985; Torres and Bergh, 1980; Torres et al., 1978a), Citrus (Torres et al., 1978b), and date palm (Torres and Tisserat, 1980).

Protein and isozyme analyses have proved particularly helpful in deducing systematic relationships between groups where morphological and cytological data have been

inconclusive. Electrophoretic studies have been used successfully to establish phylogenetic relationships in rice, wheat, barley, soybean, and cotton (Ladizinsky and Hymowitz, 1979). More recently, similar studies have been conducted with Amaranthus (Hauptli and Jain, 1984) and Capsicum (Panda et al., 1986).

CHAPTER III
PRODUCTION OF PINEAPPLE PLANTS IN VITRO

Introduction

Pineapple is routinely propagated vegetatively by means of lateral shoots, basal suckers, or crowns. Plant material is often limited, especially for the 80,000 plants/ha needed in the establishment of new plantations, and for the propagation of improved cultivars or newly discovered sports.

In vitro propagation of pineapple was first achieved in 1960 by Aghion and Beauchesne (1960), but it was not until 1974, with the simultaneous reports of Laksmi Sita et al. (1974) in India, Teo (1974) in Malaya, and Mapes (1974) in Hawaii that in vitro production of pineapple was considered as a commercial alternative. Most recent reports (Pannetier and Lanaud, 1976; Wakasa et al., 1978; Mathews and Rangan, 1979; Drew, 1980; Zepeda and Sagawa, 1981) deal only with the factors involved in the establishment of axillary buds in culture. They do not consider the number of plants regenerated or the efficiency of the system for reduced labor cost. Furthermore, high levels of variability were observed in regenerated 'Smooth Cayenne' plants by Wakasa

(1979). Such variability would compromise the commercial use of in vitro propagation of pineapple, since regenerated plants could not be assured to be clonal in nature.

This study was conducted using several species and cultivars to improve the technique of tissue culture of axillary buds for rapid pineapple propagation and to investigate the possible variation occurring in the regenerated plants.

Materials and Methods

Genotypes Cultured

Two cultivars of the common pineapple Ananas comosus (L.) Merr. were used: 'PR-1-67', a spiny commercial cultivar grown for fresh fruit and canning in Puerto Rico and 'Perolera', a smooth-leaved cultivar grown in the Northen Andes of South America and used for fresh fruit. The third genotype was Ananas bracteatus var. tricolor Smith, a small-fruited ornamental characterized by showy colored bracts and tricolored leaves (Fig. 3.6A). Two plants of PR-1-67 and 1 plant of the other 2 genotypes were used in order to determine intracultivar and intercultivar variability in response to tissue culture.

Sterilization and Initiation of Cultures

Crowns and stems were rinsed in water, defoliated and surface-sterilized by agitating in a 20% sodium hypochlorite solution with 2-3 drops of a surfactant (Tween 20 or Tween

80) for 20 min, followed by 3 rinses of 10 min each in sterile deionized water. Terminal and axillary buds were then excised aseptically and again surface-sterilized in a 2% sodium hypochlorite solution for 10 min followed by 3 more 10 min rinses in sterile deionized water (Fig. 3.1A). Buds were inoculated onto a modified Murashige and Skoog (1962) medium (Table 3.1), supplemented with 3% sucrose, 0.8% Difco Bacto-agar, 0.57 mM inositol, 1.2 uM thiamine HCl, 10.8 uM NAA and 8.8 uM BA. Growth regulators were added and the medium pH was adjusted to 5.7 prior to autoclaving at 121 ^{o}C, and 1.1 kg cm^{2} for 20 min. The medium was poured into 60 X 15 mm sterile Petri dishes for initial bud explants or into test tubes for subculture (Fig.3.1B).

Cultures were incubated at room temperature (24-27 ^{o}C) with a 16 hr photoperiod of 76 umol s^{-1} m^{-2} provided by cool-white fluorescent lamps. Explants were subcultured onto fresh medium in test tubes at 6 week intervals until adequate growth had occurred for transfer to liquid medium (Fig. 3.2).

In the experiment described, 3 buds from each of the 2 plants of 'PR-1-67' and 1 plant of 'Perolera' and Ananas bracteatus var. tricolor were established in this manner.

Culture Multiplication

Proliferating explants were multiplied in liquid cultures consisting of 50 ml of the medium described above in a 125 ml Erlenmeyer flask, maintained at 100 rpm on

gyrorotary shakers under 16 hr photoperiod (76 umol s^{-1} m^{-2}) provided by fluorescent lamps. Liquid shake cultures were subcultured at approximately 4 week intervals, when flasks had become tightly packed with plantlets (Fig. 3.3). In this way, numerous flasks of callus and regenerating plants were obtained from one original bud.

An experiment was conducted to determine the necessity of transferring the callus and plantlets to a medium without hormones to induce root formation and thus plant survival, and to evaluate the influence of plant growth regulators on the number and size of plants produced. Ten grams of an actively dividing 'Perolera' culture were inoculated into flasks containing 50 ml of basal medium with or without hormones (10.8 uM NAA and 8.8 uM BA). The 2 treatments were replicated 9 times.

Establishment in Soil

After each subculture plantlets 2.5 cm and larger were harvested. They were transferred to individual pots or to flats containing a commercial soil mixture and enclosed in plastic bags, creating a greenhouse effect (Fig. 3.4). The plants were incubated in a growth chamber at 28 ^{o}C under fluorescent lamps and gradua.ly hardened off by removing the plastic covers. Acclimated plants were transplanted to larger pots and placed in a greenhouse under a black cloth screen to prevent scorching. The screen was removed 3 weeks later and surviving plants were counted. Fertilization was

initially done biweekly with an acid-forming plant fertilizer and later on with a solution of 1/10 MS salts as apparent nutrient deficiencies developed. Phenotypic observations of the plants were made periodically.

Results and Discussion

Production of plantlets from axillary bud cultures initiated on solid medium and multiplied in liquid shaker cultures started 9 months after explanting. The total number of harvestable plantlets doubled with each monthly subculture after the 11th month (Table 3.2). From 300 to 350 plantlets could be obtained by the 13th month from a single bud culture (Table 3.3). Approximately 25 plantlets could be harvested from each 125 ml flask with each additional subculture. In all genotypes, plantlets larger than 3.0 cm had a survival rate of nearly 100% when transferred to soil.

A 2-fold increase in the total number of harvestable plantlets was obtained in 6 weeks when plant growth regulators were removed from the multiplication medium (Table 3.4; Fig. 3.5). However, after 2 subcultures (14 weeks), all plantlets had been harvested and no further callus production or multiplication occurred. Further, transferring to a medium without plant growth regulators to induce root formation was not necessary for plantlet survival. Plantlets without roots produced in multiplication medium with plant growth regulators also had a high survival rate and, while initially smaller than plantlets produced in

hormone-free medium, attained the same level of growth after 2 months in soil. The cultures periodically transferred into multiplication medium with plant growth regulators appear to be capable of indefinite multiplication. Phenotypic variation using leaf morphology as a marker was assessed in approximately 2000 regenerated plants and will be discussed in detail in Chapter VII.

Tissue culture of the species A. bracteatus var. tricolor produced regenerated plants that were either tricolor, green or albino (Fig. 3.6). This indicates the chimeral origin of this genotype. It is clear that these variegated plants should not be given formal varietal rank.

Table 3.1. Murashige and Skoog salts formulation.

Salts	mg l^{-1}
Major Salts	
NH_4NO_3	1650.00
KNO_3	1900.00
$MgSO_4.7H_2O$	370.00
$CaCl_2$	440.00
KH_2PO_4	170.00
Minor Salts	
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoSO_4.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025

Table 3.2. In vitro pineapple plantlet production for each initiated bud.

Subculture[y]	Month[x]	Mean number of plantlets harvested per flask[z]								
		Perolera			PR-1-67			PR-1-67		
		Bud 1	Bud 2	Bud 3	Bud 1	Bud 2	Bud 3	Bud 1	Bud 2	Bud 3
7	9	2.5	1.0	2.3	1.5	1.7	1.5	0	1.7	0
8	10	2.4	3.3	3.1	3.3	3.5	2.5	3.7	3.8	3.0
9	11	3.7	2.8	3.4	3.1	2.7	3.2	3.2	3.2	2.7
10	12	6.6	7.5	5.6	5.0	1.0[w]	4.1	6.1	3.8	3.8
11	13	12.8	14.8	14.4	11.2	10.5	9.9	16.2	13.8	13.8

[z]Plantlets were larger than 2.5 cm.

[y]Number of subculturing after inoculation.

[x]Number of months after culture initiation.

[w]Several flasks became contaminated during the experiment.

Table 3.3. In vitro pineapple plantlet production for each initiated bud.

Total plantlet production in 5 months of harvesting[z]				
Cultivar	Bud 1	Bud 2	Bud 3	Totals
Perolera	380	237	212	829
PR-1-67	321	42[y]	344	707
PR-1-67	309	333	112[y]	754

[z]Harvesting began 8 months after culture initiation.

[y]Several cultures became contaminated during the experiment.

Table 3.4. In vitro pineapple plantlet production in response to the presence or absence of plant growth regulators in the culture medium.

	Total number of plantlets harvested per flask[z]			
	With plant growth regulators[y]		Without plant growth regulators	
Replicate	1st harvest[x]	2nd harvest[w]	1st harvest	2nd harvest
1	20	23	7	18
2	21	30	9	17
3	16	29	12	21
4	17	25	7	17
5	21	30	11	14
6	27	28	8	16
7	18	21	7	13
8	17	24	6	14
9	20	22	10	18
Total	177	232	77	148

[z]Plantlets were 2.5 cm or larger.

[y]NAA (10.8 uM), BA (8.8 uM).

[x]Six weeks after inoculation.

[w]Fourteen weeks after inoculation.

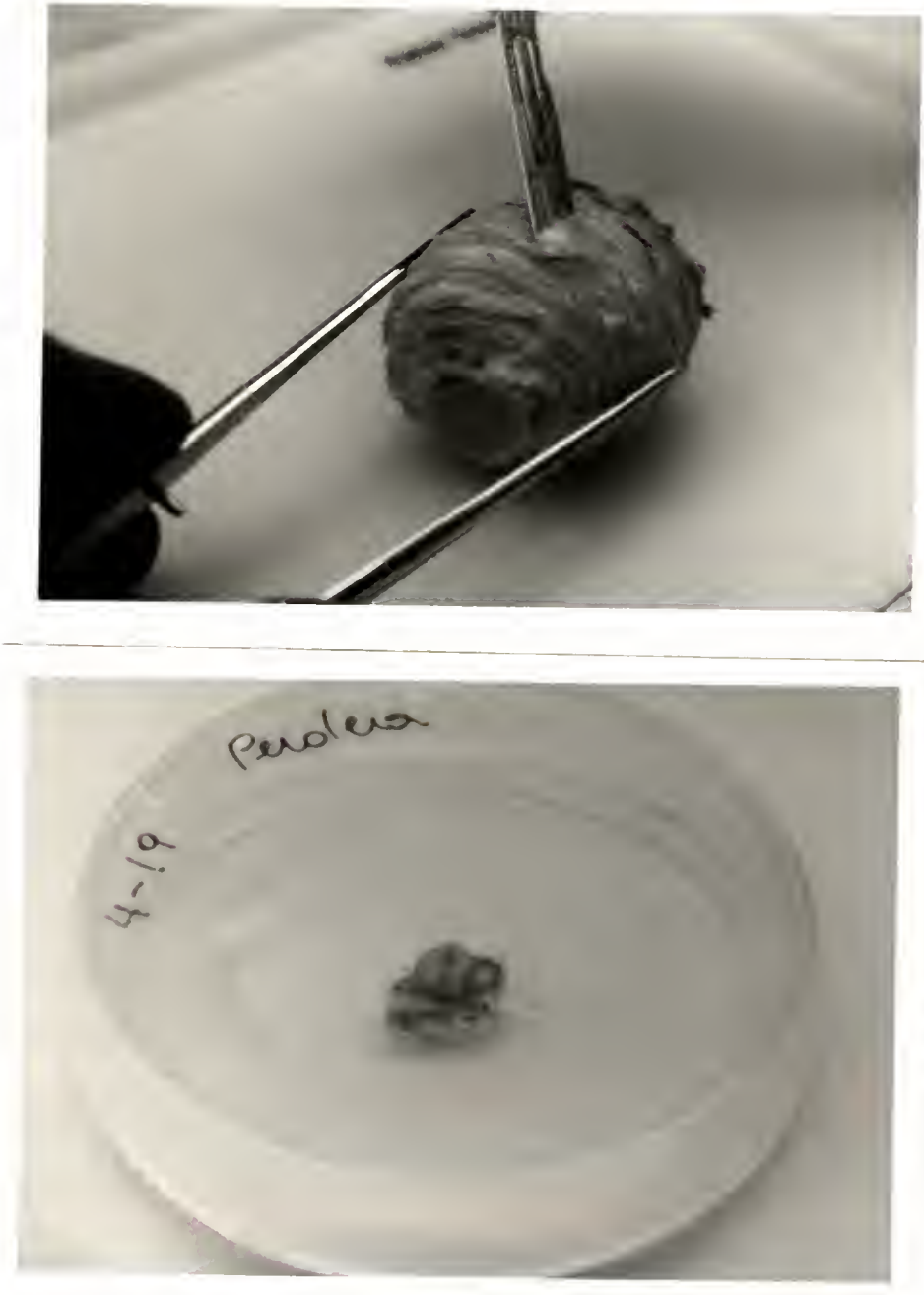


Figure 3.1. Initiation of axillary bud culture. A. Buds aseptically removed; B. Bud expansion 8 days after inoculation.

Figure 3.2. Proliferating culture 4 months after inoculation and ready to be transferred to liquid multiplication medium.

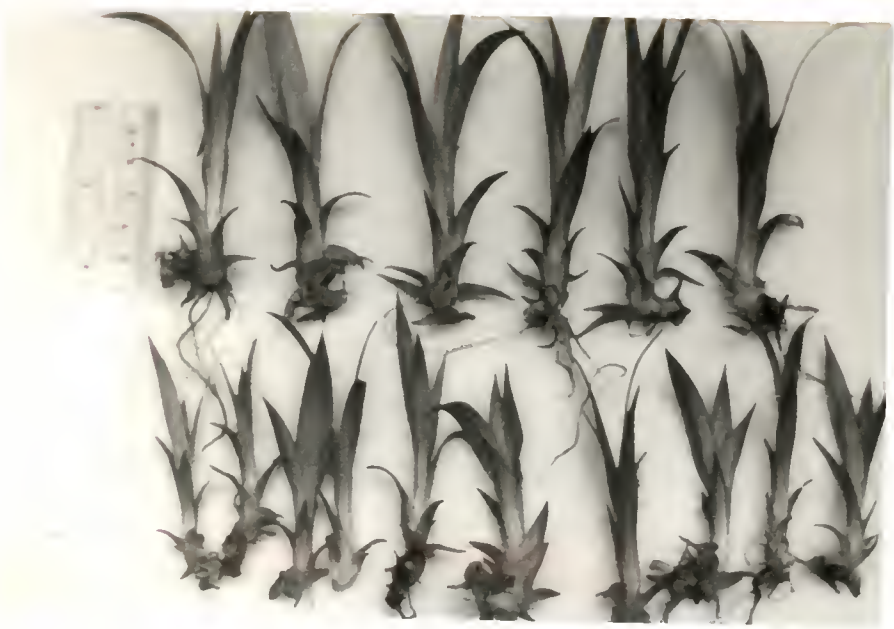
Figure 3.3. Actively dividing culture ready for subculture. Plantlets larger than 2.5 cm will be transferred to soil.

Figure 3.4. Regenerated plantlets can be established in individual 12 cm pots or more economically in flats.

A

Figure 3.5. A. Comparison of multiplication cultures with (right) and without (left) plant growth regulators.

B



C

Figure 3.5. continued. B. Plantlets produced in medium with plant growth regulators; C. Plantlets produced in medium without plant growth regulators.

A

Figure 3.6. A. Ananas bracteatus var. tricolor.

B

C

Figure 3.6. continued. B. In vitro multiplication showing resolution of chimera; C. Regenerated plants. Albino plants are incapable of survival in soil.

CHAPTER IV
ELECTROPHORETIC STUDIES IN *ANANAS*

Introduction

Electrophoretic characterization of plants based on differential patterns of protein migration is a powerful technique to separate and classify genotypes. Isozyme analysis has aided taxonomists, breeders, and physiologists in understanding the underlying causes of phenotypic variation. The plant breeder is particularly interested in classification and characterization studies in order to better utilize existing germplasm resources. The purpose of this study was to determine what enzyme and buffer systems work well in *Ananas* and which enzymes show polymorphism. The analysis was conducted with taxonomy, breeding, and cultivar improvement in mind.

No previous reports of isozyme studies have been published for pineapple. For this reason, an initial study was undertaken using a small number of *Ananas* genotypes to determine which enzyme staining systems would give well resolved banding patterns. Horizontal starch gel electrophoresis was used for isozyme characterization because it is non-toxic and yields multiple slices from a

single gel, thus allowing the rapid screening of a large number of enzyme staining systems. The procedures followed were similar to those currently being used with Citrus by Moore (personal communication, 1985). The enzyme staining systems that resulted in well resolved banding patterns were used to survey a larger number of Ananas genotypes to identify and characterize isozyme polymorphism. Genetic studies were performed using seedling populations to determine the basis of variability observed among genotypes. Information from these studies was used to formulate a system by wich cultivars, feral types, and species could be identified and distinguished, and their phenetic affinities could be established. These systems will be described in Chapters V and VI. Selected enzyme staining systems were used to determine phenotypic stability of tissue culture-regenerated plants and will be discussed in Chapter VII.

Materials and Methods

Genotypes Used for Electrophoretic Studies

A list of the genotypes used in this study is given in Table 4.1. The population included all of the species presently considered valid in the genus Ananas (Smith and Downs, 1979; Antoni, 1983) with the exception of A. fritzmuelleri, which was not available in any of the major world collections. The A. comosus cultivars surveyed represent the most important cultivars in use world-wide.

The Ananas genotypes indicated by numbers are introductions made by the author and originally established at the Facultad de Agronomia, Universidad Central de Venezuela, or material from the collection at the USDA Subtropical Horticultural Research Unit, Miami, Florida. These feral types were collected in the regions of southern Venezuela and northern Brazil, an area that has been suggested as the center of origin for the genus Ananas (Leal and Antoni, 1980). The suspected taxonomic classification of the feral types follows the introduction number. The identification was based on the botanical characters considered valid for each Ananas species by Smith and Downs (1979). High levels of phenotypic variation were present for several characters among the collected types, including presence or absence of spines, leaf color, and fruit shape, size, and color.

Genetic studies were based on isozyme analysis from both the Ananas germplasm survey and 2 seedling populations. The seedling populations were obtained from a commercial planting of 'Smooth Cayenne' and 'Cambray' growing in adjacent fields in northwestern Ecuador. Since pineapple has only been reported as self-incompatible within a cultivar and cross-compatible between cultivars (Brewbaker and Gorrez, 1967), these seedling populations were assumed to be 2 segregating populations representing reciprocal crosses between 'Smooth Cayenne' and 'Cambray'. The seeds were taken from mature fruit, rinsed in water, and air dried. Seeds were scarified by a treatment of 30-60 sec in concentrated

sulphuric acid followed by a rinse in sterile water and were planted in covered petri dishes containing a sterilized soil mixture moistened with sterile water. Periodic sprays with a 1% Captan solution were made to avoid fungal contamination. Germinated seeds were transferred to pots containing a commercial soil mix composed of sphagnum peat moss, horticultural grade vermiculite and perlite, composted pine bark, and washed granite sand. The pots were covered with polyethylene bags to prevent dessication. Transplants were initially held in a growth chamber at 27 ^{o}C with 16 hr fluorescent light (76 umol s^{-1} m^{-2}), and were taken to the greenhouse after acclimation.

All plants of the collection were kept at Gainesville, Florida, under greenhouse conditions during the cold months (Nov.-Apr.) and outside the rest of the year. Plants were kept in 25 cm pots with the same soil mixture as the seedlings and were periodically fertilized with a standard water-soluble 20-20-20 fertilizer.

Starch Gel Preparation

A 10% (w/v) starch gel consisting of a 2:1 (w/w) mixture of Sigma and Connaught hydrolyzed potato starch was used throughout this study. All the starch was from the same lot number. Thirty grams of the starch mixture along with 300 ml of a gel buffer solution (Table 4.2) were poured into a 1000 ml side arm flask. The mixture was swirled vigorously by hand and placed on a magnetic stir pad at medium speed for

10 min to assure a well-mixed suspension and to avoid the formation of lumps in the starch. The flask was then placed on a magnetic hot plate at high heat and stirred at medium speed. The solution turned translucent as it reached maximum viscosity. At this point, the flask was again swirled by hand and the cofactor NADP (10 mg dissolved in 1 ml water) was added for those staining systems requiring it. The flask was heated again until large air bubbles appeared on the surface of the solution.

The solution was degassed immediately by vacuum before it started to cool, to avoid air bubbles forming in the gel that could interfere with the migration of proteins. The vacuum source was provided by attaching an aspirator to a sink faucet and to the flask side arm. The solution was ready to be poured when no small bubbles were present.

The molds used had inside dimensions of 19 x 19 x 0.5 cm and were made from plexigas sealed with methylene chloride. Any bubbles that formed while pouring the starch solution into the molds were removed with a spatula before the gel started to harden. The gel was allowed to cool for approximately 30 min at room temperature, and then further cooled by refrigeration (4 ^{o}C) for 1 hr prior to loading the samples. Gels could be kept for 12-16 hr at room temperature before being placed in the refrigerator and they were covered with polyethylene film to prevent dessication.

Protein Extraction and Gel Loading

Leaf sap was used as the main protein source throughout this study because it was easily prepared, readily availabile, and gave good results. All leaf samples were taken from the fourth leaf at the center of the plant, counting from the first visible leaf, and were used immediately or stored in plastic bags at 4 ^{o}C for later use. Samples were used within 7 days of collection, although no decrease in enzyme activity could be detected for up to 3 weeks in storage. Leaf sap was expressed from the proximal portion of the adaxial surface of the lamina, using a pestle and a filter paper wick (Whatman No. 3, 4 x 8 mm). The white waxy epidermis and underlying layer of fibers were scraped from the leaf surface to aid in leaf sap absorption into the wick. The saturated wick was then inserted into a slit 5 cm from the top (cathodal side) of the chilled gel for anodal migrating enzymes and at 8 cm from the top for cathodal migrating enzymes, e.g. peroxidase. Once all samples had been inserted, glass rods were placed at the top and bottom of the gel to ensure good contact between wicks and gel during electrophoresis.

Pollen was collected from flowering plants just prior to anthesis, dried at room temperature for 24 hr, and frozen at -4 ^{o}C until used in electrophoretic assays. Proteins were extracted from the pollen using the method outlined by Weeden and Gottlied (1980). A small amount of frozen pollen

was placed in 0.1 ml of extraction buffer for 4 hr. The supernatant was then absorbed into the wick.

Electrophoretic Buffers and Staining Systems

Five electrophoretic buffer systems were tested: histidine citrate (H), lithium borate/tris citrate (LBTC), tris borate (TB), tris citrate (TC), and K buffer (K) [Table 4.2], as were 38 enzyme activity stains. Tested combinations are listed in Table 4.3.

The formulations used for the enzyme staining solutions were derived from several authors. Endopeptidase (E.C. 3.4.22.?) was according to Melville and Scandalios (1972). Formate dehydrogenase (E.C. 1.2.1.2) was from Wendel and Parks (1982). Creatine kinase (E.C. 2.7.3.2) and hexokinase (E.C. 2.7.1.1) were as described by Shaw and Prasad (1970). Malate dehydrogenase (E.C. 1.1.1.37) was from Cardy et al. (1981). The following were according to Vallejos (1983): adenylate kinase (E.C. 2.7.4.3), alcohol dehydrogenase (E.C. 1.1.1.1), aldolase (E.C. 4.1.2.13), alpha-amylase (E.C. 3.2.1.1), ascorbate oxidase (E.C. 1.10.3.3), catalase (E.C. 1.11.1.6), diaphorase (E.C. 1.6.4.3), fumarase (E.C. 4.2.1.1), galactose dehydrogenase (E.C. 1.1.1.48), beta-D-galactosidase (E.C. 3.2.1.23), using alpha-napthyl-D-galactopyranosidase as a substrate, glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), beta-D-glucosidase (E.C. 3.2.1.21), glutathione reductase (E.C. 1.6.4.2), isocitrate dehydrogenase (E.C. 1.1.1.42), laccase (E.C. 1.10.3.2),

lactate dehydrogenase (E.C. 1.1.1.27), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44), pyruvate kinase (E.C. 2.7.1.40), shikimate dehydrogenase (E.C. 1.1.1.25), triose phosphate isomerase (E.C. 5.3.1.1), urease (E.C. 3.5.1.5), and xanthine dehydrogenase (E.C. 1.2.1.37). In addition to these, the following staining systems were modified from Vallejos (1983) as follows:

Acid phosphatase (E.C. 3.1.3.2)
- 100 mg alpha-napthyl acid phosphatase, Na salt
- 50 mg Fast Black salt
- 100 mg 0.1 M sodium acetate, pH adjusted to 4.7 with glacial acetic acid

Alkaline phosphatase (E.C. 3.1.3.1)
- 100 mg beta-naphthylacid phosphatase, Na salt
- 100 mg Fast Blue RR salt
- 100 mg 0.1 M tris HCl, pH 8.5

Esterase (E.C. 3.1.1.2)
- 100 mg Fast Blue RR salt
- 42 ml H_2O
- 40 ml 0.2 M NaH_2PO_4
- 18 ml 0.2 M Na_2HPO_4
- 3 ml of the following solution: 1 g alpha-napthyl acetate and 1 g beta-napthyl acetate dissolved in acetone and brought to 100 ml with H_2O

Glutamate oxaloacetate transaminase (Aspartate amino-transferase, E.C. 2.6.1.1)
- 500 mg L-aspartic acid
- 70 mg alpha-keto-glutaric acid
- 50 mg pyridoxal-5'-phosphate
- 200 mg Fast Blue BB salt
- 100 ml 0.1 M tris HCl, pH 8.0

Leucine aminopeptidase (E.C. 3.4.11.1)
- 40 mg L-leucine-beta-napthylamide HCl
- 100 mg Black K salt
- 50 ml 0,2 M tris + 0.2 M maleic anhydride
- 20 ml 0.2 N NaOH
- 30 ml H_2O

Phosphoglucomutase (E.C. 2.7.5.1)

500 mg glucose-1-phosphate, Na_2 salt
20 mg NADP
30 mg MTT
4 mg PMS
100 mg $MgCl_2$
100 ml 0.1 M tris HCl, pH 8.0
40 units glucose-6-phosphate dehydrogenase

Phosphohexose isomerase (Glucophosphate isomerase, E.C. 5.3.1.9)

100 mg fructose-6-phosphate, Na_2 salt
10 mg NADP
20 mg MTT
4 mg PMS
100 mg $MgCl_2$
100 ml 0.1 M tris HCl, pH 8.0
10 units glucose-6-phosphate dehydrogenase

Malic enzyme was modified from Cardy et al. (1981); peroxidase and superoxide dismutase were modified from Moore (unpublished) and Moore and Collins (1983), as follows:

Malic enzyme (E.C. 1.1.4.0)

30 mg NADP
30 mg NBT
4 mg PMS
100 mg $MgCl_2$
20 ml 0.1 M malic acid, pH adjusted to 7.0 with NaOH
1 ml 0.2 M NaH_2PO_4
48 ml 0.2 M Na_2HPO_4
30 ml H_2O

Peroxidase (E.C. 1.11.1.7)

250 mg para-phenylenediamine
50 mg $MnSO_4$
5 ml 1.0 M sodium acetate, pH adjusted to 4.7 with glacial acetic acid
30 ml 95% ethanol
65 ml H_2O
0.5 ml 30% H_2O_2 added after above solution is poured onto gel

Superoxide dismutase (E.C. 1.15.1.1)
2 mg Riboflavin
75 mg Na_2EDTA
10 mg NBT
100 ml 0.1 M tris HCl, pH 8.0

Electrophoresis

The gel with sample wicks in place was set on top of 2 plexiglass buffer tanks. The electrode consisted of a 19 cm length of platinum wire. Five hundred milliliter of buffer solution was added to each electrode buffer tank, covering the electrode. The bridges between the electrode buffer and the gel consisted of 4-ply chromatography paper (Whatman No. 3). A glass plate was placed on top of the gel to assure good contact between the gel and electrode bridges.

Electrophoresis was carried out under constant voltage in a refrigerator at 4 ^{o}C. Power settings and running time varied with each buffer and enzyme staining system. Gels were typically run for the initial survey of buffers at 100 to 180 volts and approximately 20 milliamps for 16 to 18 hr or at 200 to 250 volts and 25 to 50 milliamps for 4 to 6 hr. Protein migration was monitored using several techniques. In the gels containing borate buffers, a light brown line was visible that migrated anodally during electrophoresis. In gels using K buffer, a sample wick saturated with bromophenol blue was used to follow the progress of electrophoresis. Protein migration was monitored in gels using histidine buffers by noting a pink color in the anode electrode buffer solution. Protein migration could also be

monitored by a decrease in current readings when using a constant voltage source during electrophoresis.

Enzyme Activity Staining

Gels were sliced horizontally into 4 slices (2 mm thick), the top slice was discarded. The gel slices were placed in plastic boxes and refrigerated until staining (usually less than 2 hr).

Staining solutions were prepared fresh, poured over the gel slice and swirled lightly to eliminate air bubbles trapped underneath. Gels were placed in the dark and observed every 15 to 30 min to follow the progress of staining. When optimum resolution was observed, the gels were rinsed in deionized water and examined. Gels were fixed for long-term storage using a (7:7:1 v/v/v) solution of methanol, deionized water, and glacial acetic acid. The gels were then wrapped in polyethylene film and refrigerated.

Analysis of Banding Patterns

The banding patterns (zymograms) were recorded by measuring the migration rate of each band. An internal standard was created in each gel system to calculate relative migration rates. The borate front produced in the TB and LBTC buffer systems and the bromophenol blue band in the K buffer systems served as an internal standard for these systems. Buffer systems that did not produce a front or a bromophenol blue band (H and TC) were evaluated by

placing a sample with a unique banding pattern in the gel, one band of which served as the internal standard. The migration of the band relative to the front (R_f value) or to the unique band (R_b value) was calculated by dividing the band migration distance by the migration distance of the internal standard.

A genetic mechanism to explain variability was hypothesized when zymogram polymorphism was observed among the genotypes. The 2 segregating populations available were used to test the genetic mechanism by comparing the observed ratios with the expected ratios using chi-square analysis. The possibility of genetic linkage was investigated by analyzing the segregation ratios at more than 1 locus. Linkage between loci could be suspected if chi-square analysis revealed a significant deviation from ratios expected with independent segregation.

Results and Discussion

Selection of Electrophoretic Buffers and Enzyme Systems Useful for Studying Isozymes in the Genus Ananas

The results of the survey testing 38 enzyme staining systems with 5 buffers can be seen in Table 4.3. Well-resolved banding patterns were obtained with 8 enzyme activity stains: isocitrate dehydrogenase, malate dehydrogenase, peroxidase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, phosphohexose isomerase, superoxide dismutase, and triose phosphate isomerase. Table 4.4 lists these

enzyme staining systems along with important electrophoretic parameters. These systems were selected for further study and are detailed below.

Twelve additional enzyme systems showed limited resolution or enzyme staining activity and were grouped into 3 categories: those with activity but no discrete banding, those with poorly resolved banding and no apparent variability, and those with poorly resolved banding but some apparent variability. Enzyme staining systems giving no discrete banding included beta-D-galactosidase, formate dehydrogenase, and urease. Systems giving poorly resolved banding with similar activity among genotypes were esterase and glutathione reductase. Staining systems giving poorly resolved banding patterns with some variable activity among genotypes included acid phosphatase, glucose-6-phosphate dehydrogenase, glutamate oxaloacetate transaminase, leucine aminopeptidase, malic enzyme, 6-phosphogluconate dehydrogenase, and shikimate dehydrogenase. Although 2 additional protein extraction buffers were tried with these enzyme staining systems, no improved resolution was observed.

No activity was detected for the following 18 enzymes: adenylate kinase, alcohol dehydrogenase, aldolase, alkaline phosphatase, alpha-amylase, ascorbic oxidase, catalase, creatine kinase, diaphorase, endopeptidase, fumarase, galactose dehydrogenase, beta-D-glucosidase, hexokinase,

laccase, lactate dehydrogenase, pyruvate kinase, and xanthine dehydrogenase.

The 8 enzyme activity stains that gave well-resolved banding patterns were used to survey the 62 genotypes listed in Table 4.1. In 5 of the staining systems, monomorphic banding patterns were obtained. The other 3 staining systems gave polymorphic banding patterns.

Monomorphic Enzymes

These enzymes included isocitrate dehydrogenase, phosphohexose isomerase, superoxide dismutase and triose phosphate isomerase. Banding phenotypes observed for these monomorphic systems are illustrated in Fig. 4.1. and are described below:

Isocitrate dehydrogenase. Two anodally migrating bands were observed with this stain, 1 at 2.0 and the other at 2.5 cm from the origin (Fig. 4.1A). The second band appeared less intense and was not always detected. No variability was observed among the genotypes studied.

6-Phosphogluconate dehydrogenase. The banding pattern obtained is shown in Fig. 4.1B. Two anodally migrating bands, 1 major and 1 minor, were observed for all genotypes investigated. The major band exhibited a mobility of 2.3 cm from the origin. The fainter minor band migrated 1.1 cm from the origin. Fainter poorly resolved bands were also present.

Phosphohexose isomerase. The banding pattern for this enzyme consisted of 3 bands (Fig. 4.1C). The first and

second bands stained more intensely and migrated at 2.3 and 2.6 cm, respectively, from the origin. The third band, which migrated at 3.0 cm, usually stained less intensely and was not always detected. Another unresolved zone of activity was present.

Superoxide dismutase. The banding pattern is illustrated in Fig. 4.2D. A monomorphic pattern consisting of 2 bands was observed for all the genotypes in this enzyme staining system. The acromatic bands migrated 3.7 and 4.7 cm from the origin.

Triose phosphate isomerase. Three well resolved bands were observed for this enzyme with relative migrations of 2.0, 2.5, and 2.8 cm from the origin (Fig. 4.2E). No variability was detected among the genotypes surveyed.

Enzyme Systems with Variable Banding Patterns

Variable banding patterns were observed for 3 enzyme staining systems: malate dehydrogenase, peroxidase, and phosphoglucomutase. Hypotheses to explain the observed variability in these enzyme systems were based on a genetic analysis of 2 segregating populations of plants from putative reciprocal crosses between 'Smooth Cayenne' and 'Cambray'. Isozyme patterns for peroxidase and phosphoglucomutase observed for other genotypes surveyed will be discussed in detail in Chapter V.

Peroxidase. Three regions, 2 cathodal (Per-1 and Per-2) and 1 anodal (Per-3), of well resolved variable banding

patterns were observed for all the genotypes tested, and are illustrated in Figs. 4.2 and 4.3. All 3 variable regions exhibited the same general banding patterns, consisting of either a single fast-migrating band (FF), a single slow-migrating band (SS), or a combination of the 2 (FS). The slow band for Per-1 was at R_f -0.26, while the fast band was at R_f -0.28. The slow band for Per-2 was at R_f -0.04 and the fast band was at R_f -0.11. The slow band for the anodally migrating Per-3 was at R_f 0.04 and the fast band at R_f 0.07. Zones of activity with no defined bands were observed between R_f 0.11 to 0.25 and 0.32 to 0.38.

The variability detected for peroxidase may be explained by a model of a monomeric enzyme system with 2 alleles (F and S) at each of 3 loci (Per-1, Per-2, and Per-3). The distal band (F) and proximal band (S) are produced by different alleles. Heterozygous genotypes would produce both a F and S band, while homozygous genotypes would produce either an FF or SS band. Progeny produced from the cross with 'Smooth Cayenne' (SS) as the seed parent gave all nonparental isozyme phenotypes (FS) for Per-1, which would result if parents homozygous (SS, FF) for different alleles at a locus were crossed. 'Cambray', the putative pollen parent of the seedlings, had a Per-1 phenotype of FF. Progeny showed a 1:1 ratio of FS and FF phenotypes for both Per-2 and Per-3, which would be expected if parents of the seedlings were homozygous (FF) and heterozygous (FS) respectively for the alleles at the loci. This was the case

with 'Smooth Cayenne' (FF) and 'Cambray' (FS) [Tables 4.5 and 4.7; Figs. 4.2 and 4.3A].

Seedlings obtained from open-pollinated crosses with 'Cambray' as the seed parent and 'Smooth Cayenne' as the putative pollen parent were expected to show a similar type of segregation, since this would be the reciprocal cross of the one described above and only the 2 cultivars were growing in the region. Instead, progeny from the 'Cambray' female parent exhibited only the 'Cambray' (FF) banding pattern for Per-1, as would be expected from a cross between 2 plants that were homozygous at this locus (Table 4.6 and Fig. 4.2). Chi-square values (Table 4.7) showed a very close fit to a 1:2:1 (FF:FS:SS) ratio for both Per-2 and Per-3, which indicates a cross between plants heterozygous at these 2 loci (Fig. 4.2). The banding patterns obtained from this progeny suggested that 'Smooth Cayenne' did not participate in the cross and that the seedlings are most likely the result of selfing.

Phosphoglucomutase. Three anodal regions (Pgm-1, Pgm-2, and Pgm-3) of well resolved variable banding patterns were observed in all the genotypes tested and are illustrated in Fig. 4.4. The banding patterns for the Pgm-1 region were unique in comparison to Pgm-2 and Pgm-3, and consisted of 5 total bands in the wide population of genotypes surveyed; F, S, M, L, or P were present in various combinations of 2 of these bands. Both Pgm-2 and Pgm-3 exhibited the same variable banding pattern consisting of either a single

fast-migrating band (FF), a single slow-migrating band (SS), or combination of the 2 (FS). Values for R_b were calculated using P.I. 115, a feral genotype exhibiting a distinctive banding pattern (Figs. 4.4 and 4.6) which served as an internal standard. The 5 bands for Pgm-1 were at R_b 0.43 (L), 0.63 (S), 0.73 (M), 0.83(F), and 1.0 (P). The slow band (S) for Pgm-2 was at R_b 0.84 and the fast band (F) was at R_b 0.98. The slow band (S) for Pgm-3 was at R_b 1.00 and the fast band (F) was at R_b 1.19.

The majority of plant species reported in the literature have contained either 1 or 2 loci governing the expression of phosphoglucomutase, with the exception of Camellia japonica, in which a third locus was reported by Wendel and Parks (1982). In the present study, it was found that the banding pattern observed in Ananas also may be explained by hypothesizing a monomeric enzyme with 3 loci (Pgm-1, Pgm-2, and Pgm-3). Locus Pgm-1 would be composed of 5 alleles, while Pgm-2 and Pgm-3 would each have 2 allelic forms.

The 2 seedling populations represent only 3 (F, M, and S) of the 5 possible Pgm-1 allelic forms. Heterozygous genotypes would show combinations of these 3 bands, i.e., FM, FS, or MS, while homozygous genotypes would produce either a FF, MM or SS band. Likewise, according to the model, the other 2 loci, Pgm-2 and Pgm-3, produce a distal band (F) and proximal band (S) as the result of different alleles. Heterozygous genotypes would produce both a F and S

band, while homozygous genotypes would produce either a FF or SS band (Tables 4.5, 4.6, and 4.7).

The 'Smooth Cayenne'-derived population showed a 1:1 ratio of phenotypes for Pgm-1 (FS:MS), Pgm-2 (FF:FS) and Pgm-3 (FS:SS), which would be expected if the parents were heterozygous and homozygous for each locus, i.e., Pgm-1: FM x SS, Pgm-2: FF x FS, and Pgm-3: SS x FS (Table 4.7). 'Smooth Cayenne' is heterozygous for Pgm-1 (FM) and homozygous for Pgm-2 (FF) and Pgm-3 (SS). Conversely, 'Cambray' is homozygous for Pgm-1 (SS) and heterozygous for Pgm-2 and Pgm-3 (FS). The segregation pattern for the 3 loci indicate that the seedlings are the results of a cross between 'Smooth Cayenne' x 'Cambray' (Figs. 4.5 and 4.6A).

The 'Cambray'-derived seedlings again exhibited only the 'Cambray' banding pattern for Pgm-1, as would be expected for progeny from parents homozygous at this locus (Table 4.6). Segregation was observed for Pgm-2 and Pgm-3, and chi-square values (Table 4.7) showed a very close fit to a 1:2:1 (FF:FS:FS) ratio, indicating a cross between 2 heterozygous types (FS). As discussed above, 'Smooth Cayenne' is heterozygous for Pgm-1 (FS) and homozygous for Pgm-2 (FF) and Pgm-3 (SS), so that it could not have been the pollen parent in this cross. Thus, the phosphoglucomutase banding patterns obtained for this population exhibited segregation ratios which corroborate the hypothesis that the seedlings are indeed the result of selfing and not outcrossing (Figs. 4.5 and 4.6B).

Self-incompatibility in pineapple is due to inhibition of pollen-tube growth in the upper third of the style. It is gametophytically controlled by a single S locus with multiple alleles (Brewbaker and Gorrez, 1967). In general, pineapples set no seed when self-pollinated, but can set seed when cultivars are crossed (Collins, 1960). Collins (1960) reports isolated self-fertile 'Smooth Cayenne' plants. Seed production was variable and germinated seed gave rise to seedy offspring. These plants exhibited very low vigor and continued selfing was not possible.

Pareja (1968) hypothesized that the frequent occurrence of seeds in 'Cambray' may be due to self-compatibility with pollination being accomplished by mites. The isozyme banding patterns displayed by the seedling population obtained from 'Cambray' comfirms the self-compatibility of this cultivar.

Malate dehydrogenase. One large anodal region consisting of many closely adjacent bands was observed for all genotypes tested and is illustrated in Fig. 4.5. Six of the bands were well resolved and migrated to 3.5, 3.8, 4.1, 4.5, 5.0, and 5.5 cm from the origin. The 2 fastest migrating bands, 5.0 and 5.5, were the only ones to show clear segregation. Banding patterns for the 'Smooth Cayenne'-derived seedlings exhibited segregation for these 2 bands (Fig. 4.7A) while no segregation (all seedlings had 2 bands) was observed for the 'Cambray'-derived seedlings (Fig. 4.7C). When the power setting during electrophoresis

was changed from 9 to 16, more separation of the bands was observed and segregation for other bands seemed to appear (Fig. 4.7B).

Malate dehydrogenase has been reported as a dimeric enzyme in numerous diverse plant species, e.g., avocado (Torres and Bergh, 1980), celery (Orton, 1983c), citrus (Torres et al., 1982), eucalyptus (Moran and Bell, 1983), and maize (Goodman and Stuber, 1983). Pollen zymograms from different cultivars, including 'Smooth Cayenne' and 'Cambray', were compared with their respective leaf zymograms to determine if intra-allelic heterodimer formation was present, as would be expected in a polymeric enzyme. Two bands were absent in pollen zymograms (Fig. 4.7D), implicating intra-allelic heterodimer formation. The presence of these interaction bands suggests that malate dehydrogenase functions as a polymeric enzyme in Ananas. However, more segregating populations must be analyzed before the genetics of this enzyme system can be clarified.

Linkage Analysis for Enzyme Loci

Linkage analysis was carried out between the peroxidase and the phosphoglucomutase loci. The results are given in Tables 4.8 and 4.9. Chi-square analysis by contingency tables showed that the observed ratios fit ratios expected for independent segregation.

Conclusions

1. Eight enzyme staining systems of the 38 investigated were found to give well-resolved banding patterns.
2. Well-resolved non-variable isozyme banding patterns were observed for isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphohexose isomerase, superoxide dismutase, and triose phosphate isomerase.
3. Well-resolved variable isozyme banding patterns were observed for malate dehydrogenase, peroxidase, and phosphoglucomutase.
4. A genetic model was hypothesized to account for the variability observed for peroxidase and phosphoglucomutase staining systems in the Ananas germplasm surveyed and in segregating seedling populations from 'Smooth Cayenne' and 'Cambray' pineapple cultivars. The model hypothesizes that both peroxidase and phosphoglucomutase function as monomeric enzymes coded for by 3 loci each: Per-1, Per-2, Per-3, Pgm-1, Pgm-2, and Pgm-3. Each locus contains 2 allelic forms, F and S, with the exception of Pgm-1, which exhibits 5 allelic forms: F, S, M, L, and P.
5. Independent segregation was found between all of the peroxidase and phosphoglucomutase loci.
6. The isozyme studies confirmed that the 2 seedling populations were the products of crosses between 'Smooth Cayenne' x 'Cambray' and 'Cambray' x self.

7. The existence of the 'Cambray' x self population calls into question belief that domestic pineapple cultivars are obligated self-incompatible.

Table 4.1. *Ananas* genotypes surveyed for isozyme banding patterns.

Species	Feral Types[z]	
A. ananassoides	046	*A. ananassoides*
A. bracteatus	064	*A. nanus*
A. comosus	072-1	*A. comosus*
A. lucidus	079	*A. ananassoides*
A. nanus	083	*A. ananassoides*
A. monstrosus	085	*A. comosus*
A. parguazensis	086	*A. comosus*
	092-1	*A. comosus*
A. comosus cultivars	092-2	*A. comosus*
Abaka	093	*A. comosus*
Black Jamaica	094	*A. comosus*
Brecheche	095	*A. comosus*
Bumanguesa	097	*A. parguazensis*
Cabeza de Mono	108	*A. parguazensis*
Cabezona	110	*A. comosus*
Cacho de Venado	115	?
Caicara	116	?
Cambray	117	?
Cumanesa	126	*A. comosus*
Dupuis Smooth	188	?
Esmeralda	189	?
Injerta	192	*A. comosus*
Maipure	193	?
Masmerah	196	*A. comosus*
Monte Oscuro	25291	*A. comosus*
Morada	487439	*A. comosus*
Nacional	487440	*A. comosus*
Negrita	487442	*A. comosus*
Panare	487443	*A. comosus*
Perola	487444	*A. comosus*
Perolera		
Pina de Brazil		
PR-1-67		
Queen		
Red Spanish		
Rondon		
Smooth Cayenne		
Valera Amarilla		

[z]Numbers correspond to introductions followed by the suspected taxonomical classification. "?" indicates that the botanical characteristics of the plant do not fit any of the presently described species.

Table 4.2. Electrophoretic buffer systems used for isozyme detection in Ananas.

Buffer system[z]	Electrode buffer[y]	Gel buffer[y]
H	0.065 M histidine 0.02 M citric acid pH adjusted to 5.7 with citric acid	0.009 M histidine 0.003 M citric acid pH adjusted to 5.7 with citric acid
K	0.18 M trizma base 0.1 M boric acid 0.004 M Na_2EDTA pH 8.6	0.045 M trizma base 0.025 M boric acid 0.001 M Na_2EDTA pH 8.6
LBTC	0.016 M LiOH 0.192 M boric acid pH 7.2	0.0016 M LiOH 0.019 M boric acid 0.007 M citric acid 0.046 M trizma base pH 7.7
TB	0.038 M trizma base 0.002 M citric acid pH 8.6	0.03 M boric acid pH 8.5
TC	0.05 M trizma base 0.016 M citric acid pH adjusted to 7.0 with citric acid	0.017 M trizma base 0.005 M citric acid pH adjusted to 7.0 with citric acid

[z]H = Histidine citrate (Cardy et al., 1981); K = K buffer (Loukas and Pontikis, 1979); LBTC = Lithium borate/Tris citrate (modified Scandalios, 1969); TB = Tris borate; TC = Tris citrate (Moore, unpublished).

[y]10 mg NADP was added to buffer in cathodal tank and to gel buffer when staining for enzyme system that required NADP as a cofactor.

Table 4.3. Enzyme activity stains and buffer systems tested for isozyme detection in Ananas.

Enzyme activity stain	Buffer system[z]				
	LBTC	H	TC	TB	K
Acid phosphatase	-	+	-	-	+
Adenylate kinase	-	-	-	-	*
Alcohol dehydrogenase	-	-	-	-	*
Aldolase	-	-	-	-	*
Alkaline phosphatase	-	-	-	-	*
alpha-Amylase	-	-	-	-	*
Ascorbate oxidase	-	-	-	-	*
Catalase	-	-	-	-	*
Creatine kinase	-	-	-	-	*
Diaphorase	-	-	-	-	*
Endopeptidase	-	-	-	-	*
Esterase	-	-	-	+	+
Formate dehydrogenase	+	-	-	-	*
Fumarase	-	-	-	-	*
Galactose dehydrogenase	-	-	-	-	*
beta-D-Galactosidase	-	-	-	-	*
Glucose-6-phosphate dehydrogenase	-	+	-	-	++
beta-D-Glucosidase	-	-	-	-	*
Glutamate oxalloacetate transaminase	++	-	-	-	++
Glutathione reductase	+	-	+	-	*
Hexokinase	-	-	-	-	*
Isocitrate dehydrogenase	++	+++	-	-	++
Laccase	-	-	-	-	*
Lactate dehydrogenase	-	-	-	-	*
Leucine aminopeptidase	-	-	-	+	*
Malate dehydrogenase	++	+++	++	++	++
Malic enzyme	+	-	-	+	*
Peroxidase	++	++	+	++	+++
Phosphoglucomutase	-	+++[y]	++	++	*
6-phosphogluconate dehydrogenase	+	+++	+	+	++
Phosphohexose isomerase	++	+++	++	++	++
Piruvate kinase	-	-	-	-	*
Shikimate dehydrogenase	++	-	-	-	++
Superoxide dismutase	++	-	-	-	+++
Superoxide dismutase + KCN	++	-	-	+	*
Triose phosphate isomerase	-	-	-	-	+++
Urease	+	-	-	+	*
Xanthine dehydrogenase	-	-	-	-	*

[z] - = no activity; + = poor migration or resolution; ++ = fair migration or resolution; +++ = well resolved bands; * = assay not performed.

[y] Modified to 0.065 M, pH 6.3.

Table 4.4. Well resolved enzyme activity staining systems and electrophoretic parameters used in Ananas.

Enzyme system	Electrophoretic conditions				
	Buffer system	Volts[z]	Milliamps	Total power	Time (hr)
Isocitrate dehydrogenase	H[y]	300	45	13.50	3.5
Malate dehydrogenase	H	300	40	12.00	4.0
Peroxidase	K	250	25	6.25	5.0
Phosphoglucomutase	H[y]	300	45	13.50	3.5
6-Phosphogluconate dehydrogenase	H	300	40	12.00	4.0
Phosphohexose isomerase	H[x]	300	45	13.50	4.0
Superoxide dismutase	K	250	34	8.50	4.0
Triose phosphate isomerase	K	250	25	6.25	4.0

[z]Gels were run at constant voltage, and voltage was changed during electrophoresis as milliamps increased to maintain the total power indicated.

[y]pH modified to 6.3.

[x]1.5 g l^{-1} Na_2EDTA added to the buffer to reduce the dark background in gels.

Table 4.5. Peroxidase and phosphoglucomutase banding patterns observed for seedlings derived from an open-pollinated 'Smooth Cayenne' fruit.

	Isozyme genotypes					
Seedlings	Per-1	Per-2	Per-3	Pgm-1	Pgm-2	Pgm-3
1	FS	FS	FF	MS	FS	FS
2	FS	FF	FS	FS	FS	FS
3	FS	FF	FF	FS	FS	SS
4	FS	FF	FS	MS	FF	SS
5	FS	FS	FS	FS	FS	SS
6	FS	FS	FF	MS	FS	FS
7	FS	FF	FS	MS	FF	SS
8	FS	FS	FF	MS	FS	SS
9	FS	FS	FS	MS	FS	SS
10	FS	FF	FF	FS	FS	SS
11	FS	FS	FF	MS	FS	FS
12	FS	FS	FS	MS	FS	SS
13	FS	FF	FS	FS	FS	SS
14	FS	FF	FS	FS	FF	SS
15	FS	FS	FS	MS	FS	SS
16	FS	FF	FF	FS	FS	SS
17	FS	FF	FF	FS	FS	SS
18	FS	FS	FF	FS	FF	FS
19	FS	FF	FS	MS	FS	SS
20	FS	FS	FF	MS	FS	SS
21	FS	FS	FF	FS	FS	SS
22	FS	FF	FF	FS	FF	FS
23	FS	FS	FS	MS	FF	SS
24	FS	FF	FF	FS	FF	FS
25	FS	FS	FF	MS	FF	FS
26	FS	FS	FF	FS	FF	FS
27	FS	FS	FF	MS	FF	FS
28	FS	FS	FF	MS	FF	SS

Table 4.6. Peroxidase and phosphoglucomutase banding patterns observed for seedlings derived from an open-pollinated 'Cambray' fruit.

Seedlings	Isozyme genotypes					
	Per-1	Per-2	Per-3	Pgm-1	Pgm-2	Pgm-3
1	FF	FS	FS	SS	FS	FS
2	FF	FS	FF	SS	FS	FF
3	FF	FS	FS	SS	FF	FF
4	FF	FS	SS	SS	SS	FS
5	FF	FF	SS	SS	FF	FS
6	FF	FS	FF	SS	SS	SS
7	FF	FS	SS	SS	FS	FS
8	FF	SS	FS	SS	FS	FS
9	FF	FF	SS	SS	FS	FS
10	FF	FS	SS	SS	SS	FS
11	FF	FS	FS	SS	SS	SS
12	FF	SS	FF	SS	FF	FS
13	FF	FF	FS	SS	FF	SS
14	FF	FF	FS	SS	FS	FS
15	FF	FS	FF	SS	SS	SS
16	FF	SS	FS	SS	FS	FS
17	FF	FF	FS	SS	FF	SS
18	FF	SS	FS	SS	FF	FS
19	FF	FS	FS	SS	FS	FF
20	FF	SS	FS	SS	FS	FF

Table 4.7. Genotypic ratios and chi-square goodness of fit values for 6 isozyme loci in pineapple.

Loci investigated[z]	Segregation classes				Test ratio	X^2	P value
	FF	FS	MS	SS			
Smooth Cayenne seedlings							
Per-1 (SSxFF)	-	28	-	-	--		
Per-2 (FFxFS)	12	16	-	-	1:1	0.571	.50-.25
Per-3 (FFxFS)	17	11	-	-	1:1	1.285	.50-.25
Pgm-1 (FMxSS)	-	13	15	-	1:1	0.142	.75-.50
Pgm-2 (FFxFS)	10	18	-	-	1:1	2.285	.25-.10
Pgm-3 (SSxFS)	-	10	-	18	1:1	2.285	.25-.10
Cambray seedlings							
Per-1 (FFxFF)	20	-	-	-	--		
Per-2 (FSxFS)	5	10	-	5	1:2:1	0.000	1.000
Per-3 (FSxFS)	4	11	-	5	1:2:1	0.100	.90-.75
Pgm-1 (SSxSS)	-	-	-	20	--		
Pgm-2 (FSxFS)	6	9	-	5	1:2:1	0.100	.90-.75
Pgm-3 (FSxFS)	4	11	-	5	1:2:1	0.100	.90-.75

[z]Seedlings from putative crosses of 'Smooth Cayenne' x 'Cambray' and 'Cambray' x self. Parentheses indicate allelic combinations for parents at the locus.

Table 4.8. Segregation ratios and chi-square goodness of fit values for independent inheritance of peroxidase and phosphoglucomutase loci in 'Smooth Cayenne'-derived seedlings.

Loci pair	Segregation classes (no. of plants obs.)				Test ratio	X^2 value	P value
Per-1/Per-2	-[z]						
Per-2/Per-3	FS/FS(5)	FS/FF(11)	FF/FS(6)	FF/FF(6)	1:1:1:1	0.377	.75-.50
Per-2/Pgm-1	FS/MS(12)	FS/FS(5)	FF/MS(3)	FF/FS(8)	1:1:1:1	3.447	.10-.05
Per-2/Pgm-2	FS/FS(10)	FS/FF(6)	FF/FS(8)	FF/FF(4)	1:1:1:1	0.029	.90-.75
Per-2/Pgm-3	FS/SS(10)	FS/FS(7)	FF/SS(8)	FF/FS(3)	1:1:1:1	0.120	.75-.50
Per-3/Pgm-1	FS/MS(7)	FS/FS(4)	FF/MS(9)	FF/FS(9)	1:1:1:1	0.110	.75-.50
Per-3/Pgm-2	FS/FS(8)	FS/FF(3)	FF/FS(10)	FF/FF(7)	1:1:1:1	0.120	.75-.50
Per-3/Pgm-3	FS/SS(10)	FS/FS(1)	FF/SS(8)	FF/FS(9)	1:1:1:1	3.846	.05-.02
Pgm-1/Pgm-2	MS/FS(11)	MS/FF(5)	FS/FS(8)	FS/FF(4)	1:1:1:1	0.085	.90-.75
Pgm-1/Pgm-3	MS/SS(10)	MS/FS(5)	FS/SS(8)	FS/FS(5)	1:1:1:1	0.013	.95-.90
Pgm-2/Pgm-3	FS/SS(14)	FS/FS(4)	FF/SS(4)	FF/FS(6)	1:1:1:1	2.528	.25-.10

[z]"-" indicates no segregation.

Table 4.9. Segregation ratios and chi-square goodness of fit values for independent inheritance of peroxidase and phosphoglucomutase loci in 'Cambray'-derived seedlings.

Loci pair	Segregation classes (no. of plants obs.)				Test ratio	X^2 value	P value
Per-1/Per-2	-[z]						
Per-2/Per-3	FF/FF(0)	FF/FS(3)	FF/SS(2)	FS/FF(3)	1:2:1:		
	FS/FS(4)	FS/SS(3)	SS/FF(1)	SS/FS(4)	1:2:1:		
	SS/SS(0)				1:2:1	4.300	.50-.25
Per-2/Pgm-1	-						
Per-2/Pgm-2	FF/FF(3)	FF/FS(2)	FF/SS(0)	FS/FF(1)	1:2:1:		
	FS/FS(4)	FS/SS(5)	SS/FF(2)	SS/FS(3)	1:2:1:		
	SS/SS(0)				1:2:1	8.320	.10-.05
Per-2/Pgm-3	FF/FF(0)	FF/FS(3)	FF/SS(2)	FS/FF(3)	1:2:1:		
	FS/FS(4)	FS/SS(3)	SS/FF(1)	SS/FS(4)	1:2:1:		
	SS/SS(0)				1:2:1	4.300	.50-.25
Per-3/Pgm-1	-						
Per-3/Pgm-2	FF/FF(1)	FF/FS(1)	FF/SS(2)	FS/FF(4)	1:2:1:		
	FS/FS(6)	FS/SS(1)	SS/FF(1)	SS/FS(2)	1:2:1:		
	SS/SS(2)				1:2:1	3.517	.50-.25
Per-3/Pgm-3	FF/FF(1)	FF/FS(1)	FF/SS(2)	FS/FF(3)	1:2:1:		
	FS/FS(5)	FS/SS(3)	SS/FF(0)	SS/FS(5)	1:2:1:		
	SS/SS(0)				1:2:1	6.000	.25-.10
Pgm-1/Pgm-2	-						
Pgm-1/Pgm-3	-						
Pgm-2/Pgm-3	FF/FF(1)	FF/FS(3)	FF/SS(2)	FS/FF(3)	1:2:1:		
	FS/FS(6)	FS/SS(0)	ss/FF(0)	SS/FS(2)	1:2:1:		
	SS/SS(3)				1:2:1	7.148	.25-.10

[z]"-" indicates no segregation.

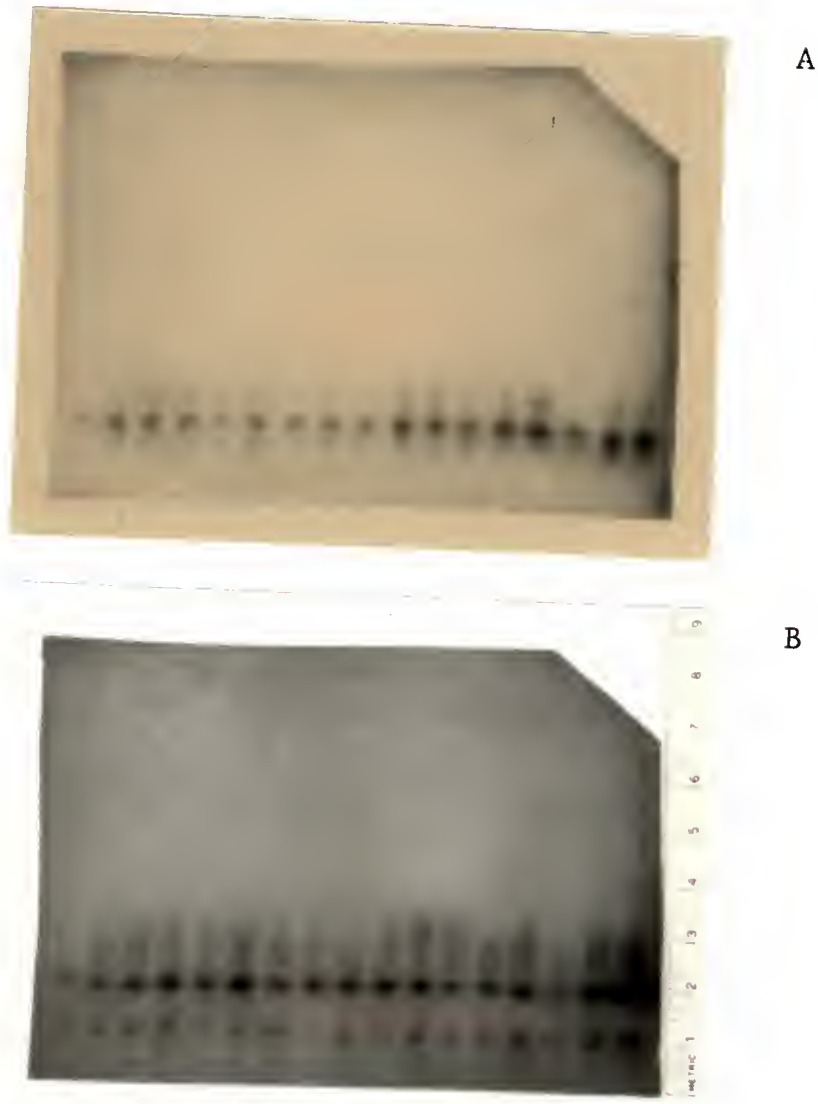


Figure 4.1. Non-variable isozyme banding patterns observed for Ananas genotypes. A. Isocitrate dehydrogenase; B. 6-Phosphogluconate dehydrogenase. Sample origin is at the bottom of each photograph and band migration is anodal.

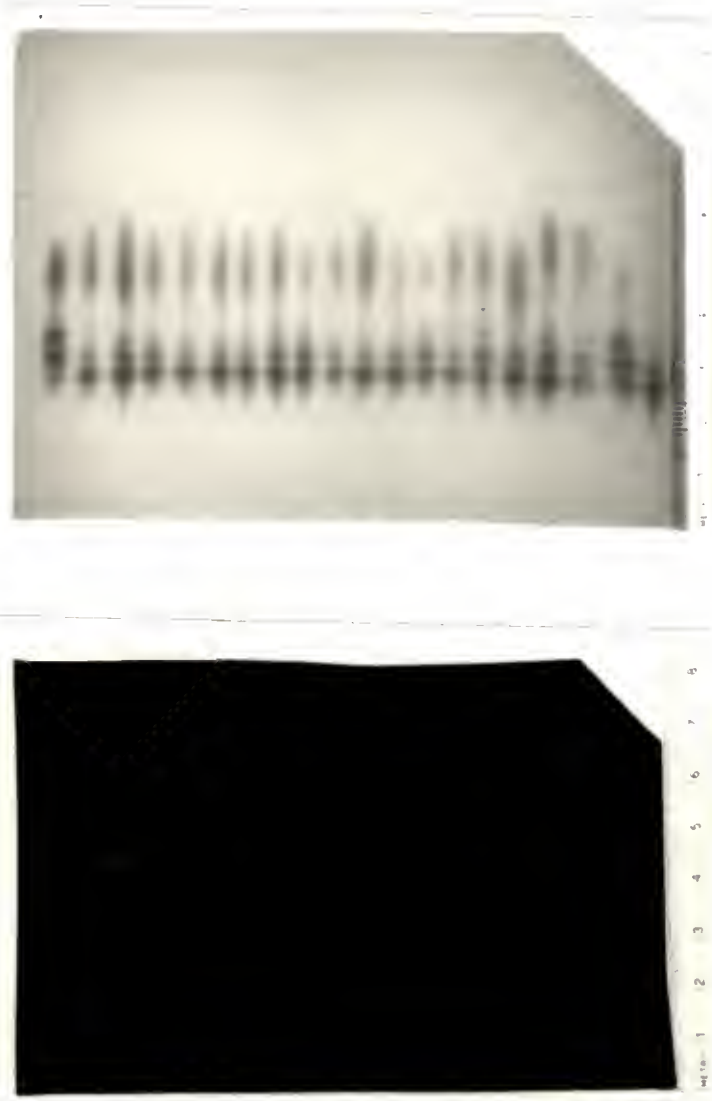


Figure 4.1. continued. C. Phosphohexose isomerase; D. Superoxide dismutase.

Figure 4.1. continued. E. Triose phosphate isomerase.

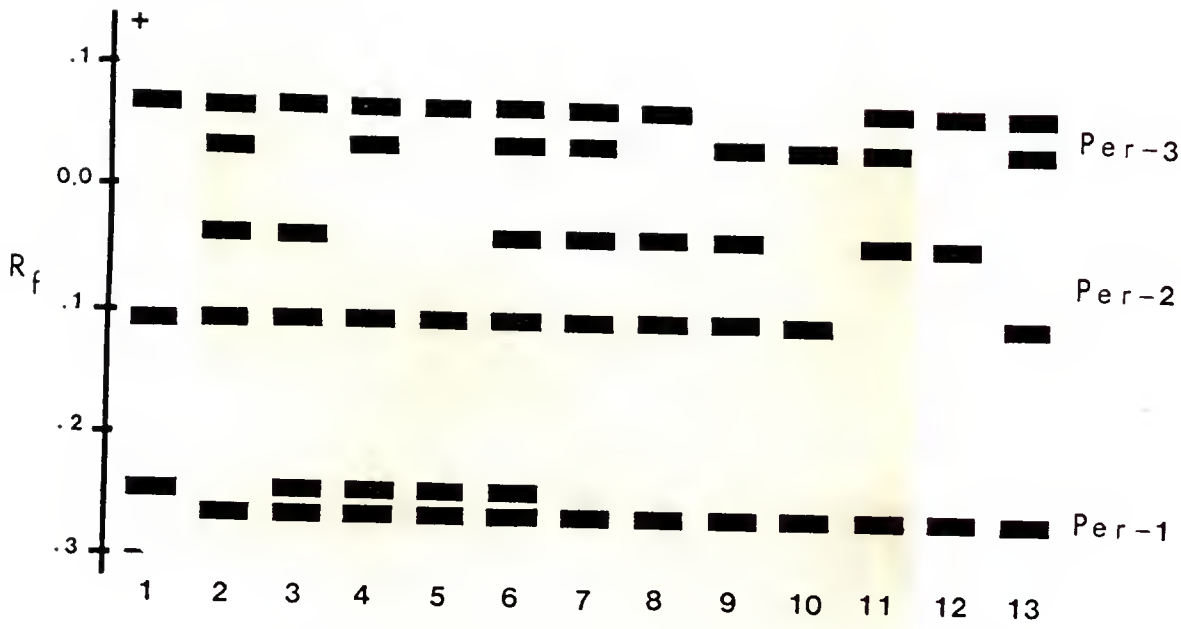
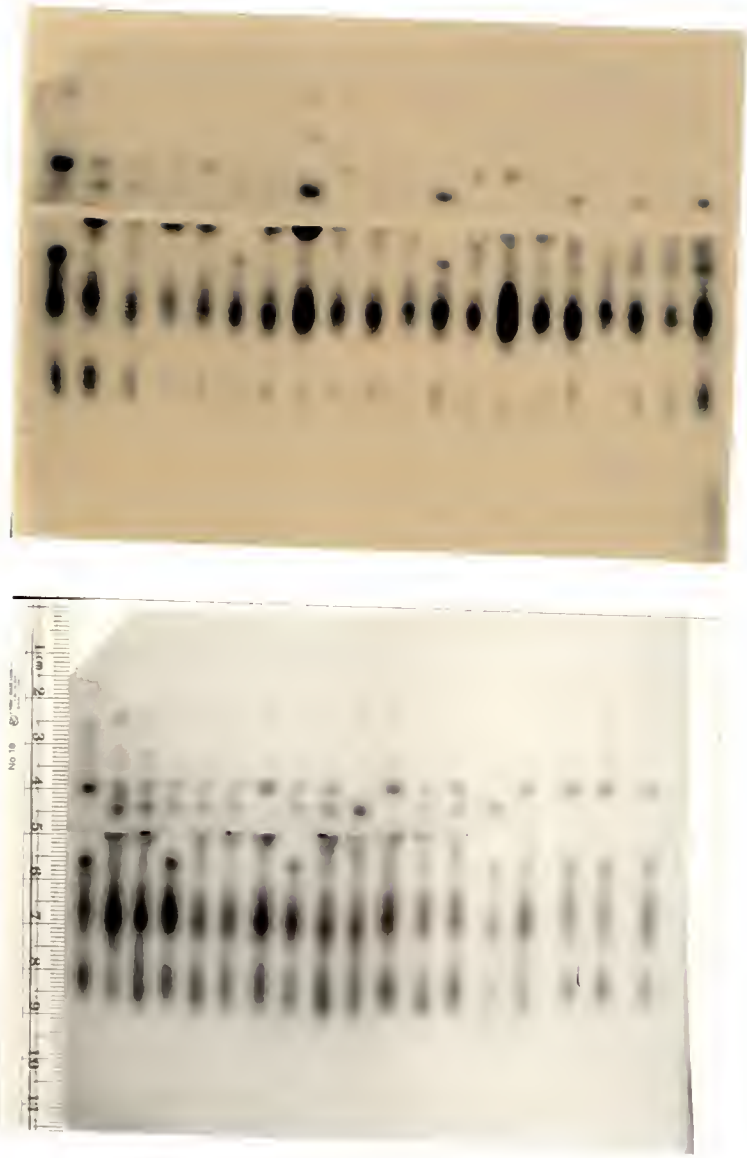


Figure 4.2. Schematic representation of peroxidase isozymes in 2 pineapple segregating populations. 1. 'Smooth Cayenne'; 2. 'Cambray'; 3-6. Hybrids of 'Smooth Cayenne' x 'Cambray' cross; 7-13. Hybrids of 'Cambray' x self cross.



A

B

Figure 4.3. Peroxidase banding patterns observed in pineapple. A. From right to left, 'Smooth Cayenne','Cambray', and progeny from the putative cross 'Smooth Cayenne' x 'Cambray'; B. 'Smooth Cayenne', 'Cambray', and progeny from the putative cross 'Cambray' x self. Sample origin is at the middle of each photograph and band migration is both anodal and cathodal.

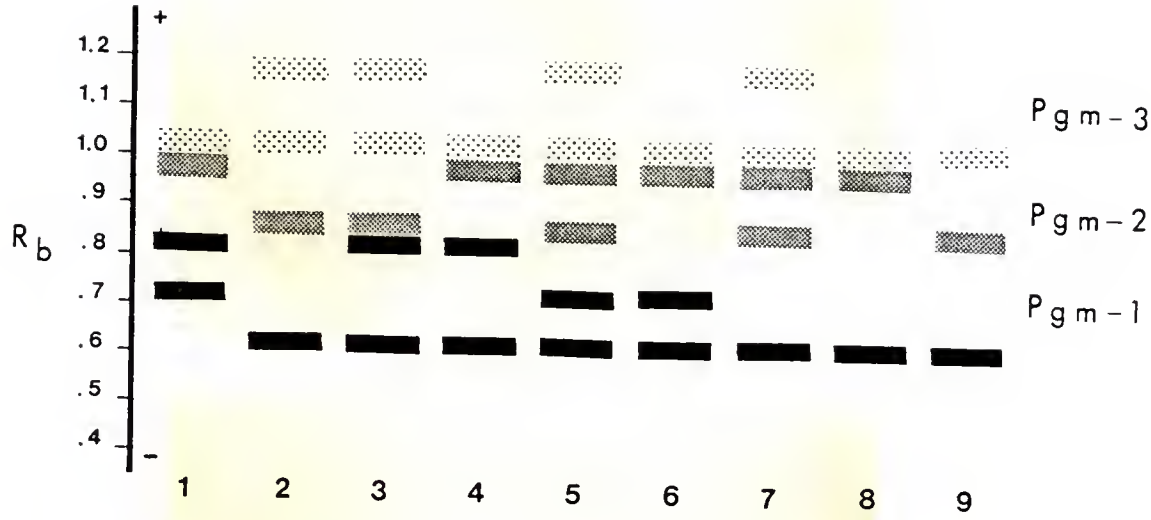


Figure 4.4. Schematic representation of phosphoglucomutase isozyme in Ananas. 1. P.I. 115; 2. 'Maipure'; 3. 'Smooth Cayenne'; 4. P.I. 072; 5. P.I. 085; 6. 'Black Jamaica'; 7. P.I. 095; 8. P.I. 27281; 9. 'Brecheche'. Black bands = Pgm-1; cross-hatched bands = Pgm-2; dotted bands = Pgm-3.

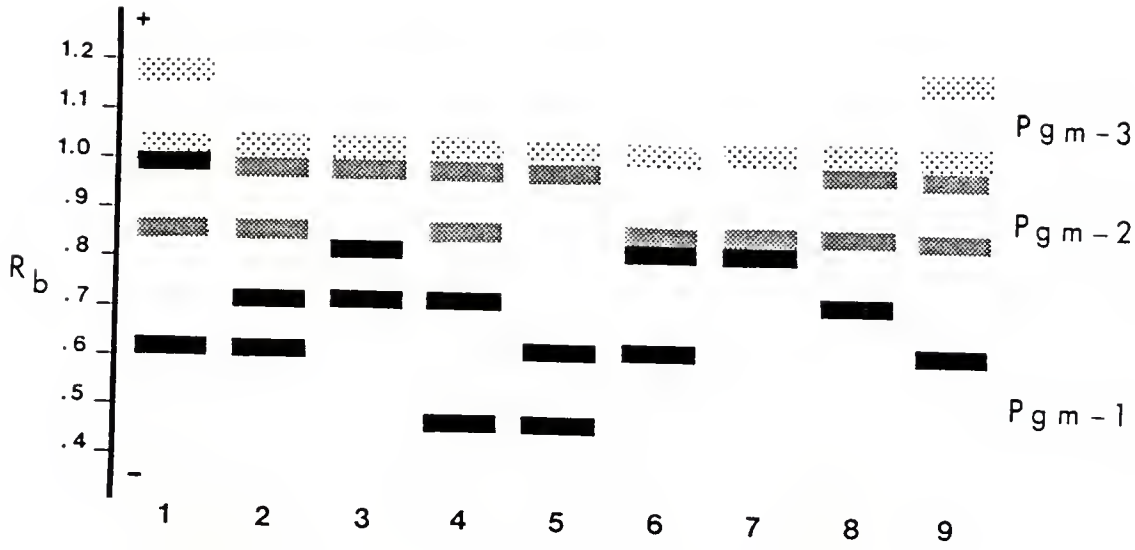


Figure 4.5. Schematic representation of phosphoglucomutase isozyme in 2 pineapple segregating populations. 1. 'Smooth Cayenne'; 2. 'Cambray'; 3-6. Hybrids of 'Smooth Cayenne' x 'Cambray' cross; 7-9. Hybrids of 'Cambray' x self cross.

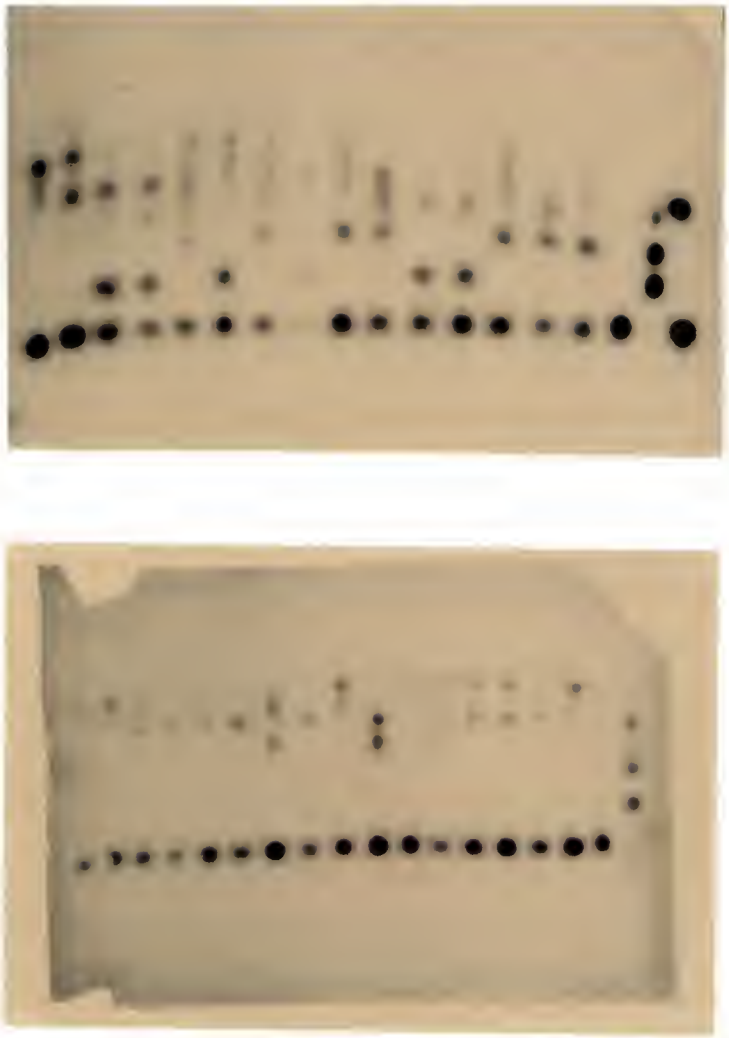


Figure 4.6. Phosphoglucomutase banding pattern in pineapple. A. From right to left, P.I. 115, 'Smooth Cayenne', 'Cambray', and progeny from the putative cross 'Smooth Cayenne' x 'Cambray'; B. 'Smooth Cayenne', 'Cambray', and progeny from the putative cross 'Cambray' x self. Sample origin is at the bottom of each photograph and band migration is anodal.

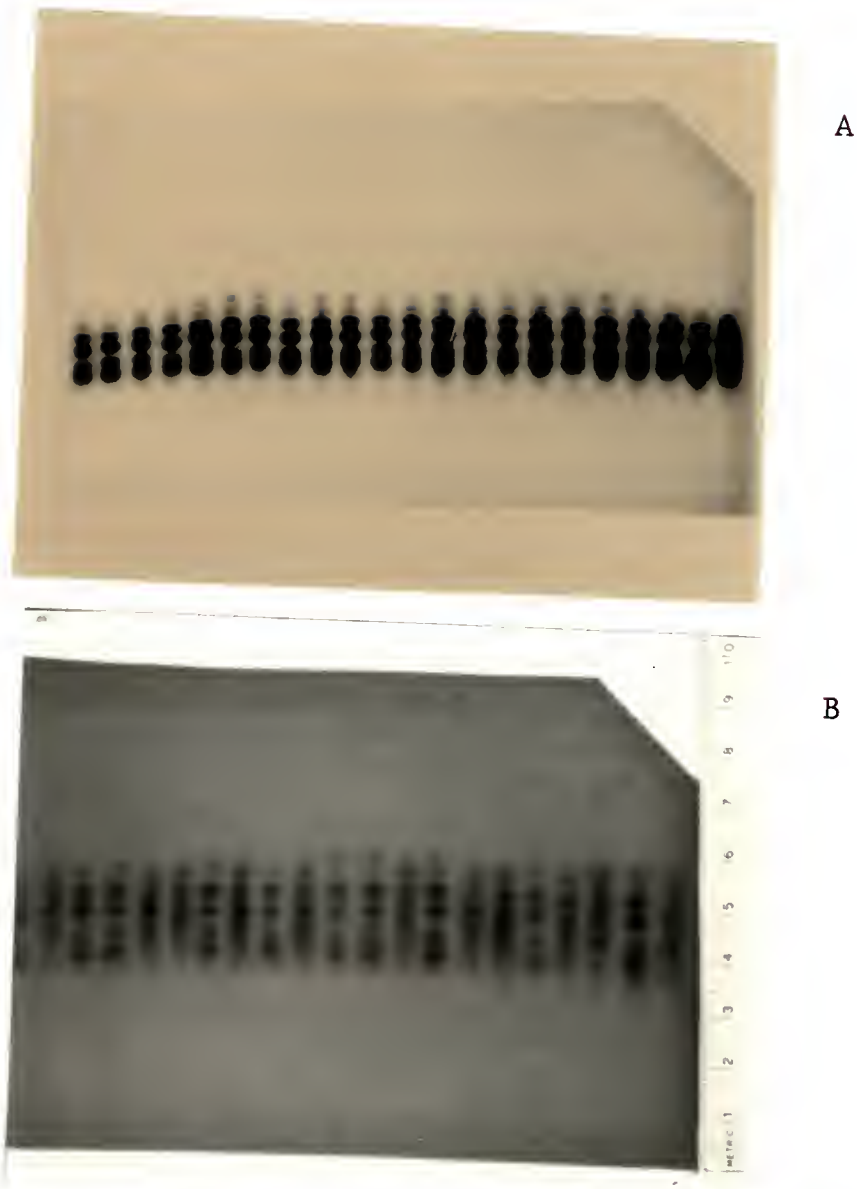
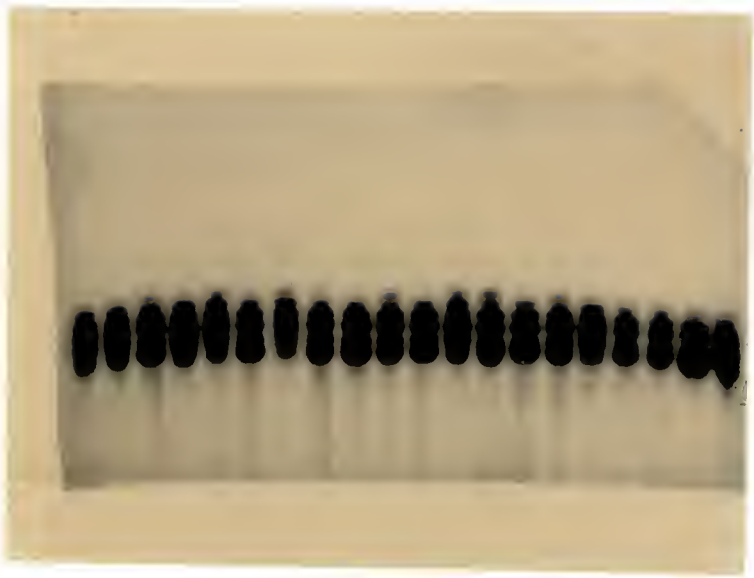
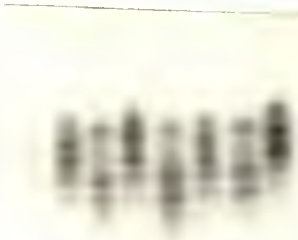


Figure 4.7. Malate dehydrogenase patterns observed in pineapple. A. and B. Gels run at different power settings (9 and 16), from right to left, 'Smooth Cayenne', 'Cambray', and seedlings from the putative cross 'Smooth Cayenne' x 'Cambray'. Sample origin is at the bottom of each photograph and band migration is anodal.



C



D

Figure 4.7. continued. C. Progeny from the putative cross 'Cambray' x self; D. Pollen and leaf, from right to left, 'Smooth Cayenne', 'Brecheche', and 'Cambray'.

CHAPTER V
IDENTIFICATION OF *ANANAS* GENOTYPES BY ISOZYME PHENOTYPES

Introduction

The utilization of isozyme techniques for cultivar identification requires high levels of clearly recognizable inter-cultivar isozyme polymorphism, preferably with monomorphic cultivar phenotypes. The phenotypes should be stable over a variety of environmental and developmental conditions. The techniques used to characterize the phenotypes should be simple and efficient.

Ananas comosus appears to be an ideal candidate for electrophoretic characterization using isozyme polymorphisms. The self-incompatible and outcrossing nature of the species would be expected to generate high levels of inter-specific genetic variation, while intra-clonal variability would be expected to be relatively low due to the apomictic nature of pineapple.

The taxonomy and origin of the genus *Ananas* has been the subject of much debate and speculation. Species are generally distinguished by the presence or absence and direction of leaf spines, petal morphology, and fruit size.

The purpose of this study was to electrophoretically characterize the genus Ananas using isozyme polymorphisms with emphasis on gaining information on the origin of the commercially important pineapple cultivars of the world.

Materials and Methods

Starch gels were prepared and electrophoresis was conducted as described in Chapter IV. Two buffer systems were used for electrophoresis, a histidine system at pH 6.3 and a K buffer at pH 8.6 (Table 4.2). The histidine buffer was used to assay for phosphoglucomutase activity and the K buffer for peroxidase activity. At least 2 plants of each genotype (Table 4.1) were examined electrophoretically for peroxidase and phosphoglucomutase polymorphisms, except that no peroxidase determination was made for the feral types. Assays were repeated until consistent results were obtained. The banding patterns were recorded using both schematic drawings and photographs. Band migration distances were based on R_f and R_b values as calculated in Chapter IV.

Results and Discussion

Considerable isozyme polymorphism was observed among the genotypes examined for both peroxidase and phosphoglucomutase. In both systems, bands were characterized for each locus according to inheritance studies discussed in Chapter IV. Peroxidase showed either a

single or a double banded pattern at 2 migrating regions, corresponding to the Per-2 and Per-3 loci. No clear bands were present for Per-1. Phosphoglucomutase exhibited the most useful isozyme polymorphisms. Bands corresponding to the Pgm-1 locus gave consistent and clear resolution but overlapping bands corresponding to Pgm-2 and Pgm-3 loci complicated the characterization of certain genotypes.

Cultivar Identification

Peroxidase. Limited isozyme polymorphism was detected for peroxidase. A total of 7 different banding patterns was observed and designated A_1 through A_7 (Table 5.1; Figs. 5.1 and 5.2). Patterns A_3, A_4, and A_7 were unique to 'Negrita', 'Pina de Brazil', and 'Maipure' respectively. 'Panare' and 'Queen' shared pattern A_6, while the other cultivars showed patterns A_1, A_2 or A_5 (Table 5.2).

Phosphoglucomutase. Eleven different phosphoglucomutase banding patterns were identified and designated B_1 through B_{11} (Tables 5.1 and 5.2; Figs. 5.1 and 5.2). Unique banding patterns were observed for 'Black Jamaica', 'Rondon', 'Valera Amarilla', 'Cacho de Venado', and 'Cabezona'. These patterns were B_3, B_4, B_7, B_8, and B_{10} respectively. The rest of the cultivars were distributed over the patterns B_1, B_2, B_5, B_6, B_9, and B_{11} (Table 5.3).

The polymorphic enzyme systems of peroxidase and phosphoglucomutase were very useful for cultivar

classification. Using only the peroxidase or the phosphoglucomutase isozyme system, 8 cultivars could be distinguished by their unique banding patterns (Tables 5.2 and 5.3). However, using information combined from both the peroxidase and phosphoglucomutase isozyme systems, 20 unique classes of banding patterns could be distinguished. These classes permitted the identification of 15 cultivars out of the total 27 examined (Table 5.4).

The origin and exact relationship of many of the pineapple cultivars studied remains unclear. A wide variety of clones and cultivars exists because of the prevalence of inter-clonal fertility and apomixis accompanied by self-incompatibility. Certain pineapple cultivars and clones exhibit high rates of mutation and many unique sports exist; e.g., 'Smooth Cayenne', the most economically important cultivar, is really a group of clones (Collins, 1960). These factors considerably complicate characterization and classification schemes.

Based on the peroxidase and phosphoglucomutase isozyme banding patterns, it appears that there is a very close relationship among many of the cultivars tested. 'PR-1-67', a reputed hybrid widely grown in Puerto Rico, was obtained from an open-pollinationed seed of 'Red Spanish' growing adjacent to a field of 'Smooth Cayenne' (Ramirez et al., 1970 and 1972). The parental relationship of these 2 cultivars to 'PR-1-67' is corroborated by their isozyme banding patterns (Table 5.1). 'Esmeralda', a cultivar grown

in Mexico (Samuels, 1970), and 'Dupuis Smooth' are presumed to be sports of 'Smooth Cayenne'. The 3 cultivars exhibited the same banding pattern, indicating a common origin. 'Cumanesa' is very similar morphologically to 'Red Spanish' and is believed to be a sport of the latter (Leal and Antoni, 1980b). The identical banding pattern showed by the 2 cultivars suggests that 'Cumanesa' could indeed have originated as a sport of 'Red Spanish'.

'Bumanguesa' and 'Perolera' are the 2 cultivars commonly grown in the Andean regions of Venezuela and Colombia. Both cultivars have similar morphological characteristics of plant growth and differ only in mature fruit color. 'Bumanguesa' produces a bright red fruit, while 'Perolera' produces a yellow fruit. Because of the similarities of these 2 cultivars, Leal et al. (1979) hypothesized that 'Bumanguesa' was a sport of 'Perolera'. However, the peroxidase and phosphoglucomutase banding pattern observed for these 2 cultivars do not indicate a close relationship, unless the mutation involved the Per-2, Per-3, and Pgm-3 loci (Table 5.1).

The cultivars 'Queen' and 'Panare', which represent the "Queen" group of pineapples, exhibited identical isozyme banding patterns for both peroxidase and phosphoglucomutase. They also are very similar morphologically. This is remarkable since cultivars belonging to this horticultural group are grown commercially only in South Africa and Australia (Samuels, 1970). 'Panare' was described by Leal et

al. (1979) from a clone being cultivated in southern Venezuela (Bolivar State). Local people reported that the original plants were "wild" types found growing nearby. Since the area including southern Venezuela and northern Brazil has been suggested as the center of origin for the Ananas genus (Leal and Antoni, 1980a) it seems likely that the "Queen" group probably was derived from 'Panare'-like wild types. This finding confirms the presence of "Queen" types growing in the Western Hemisphere.

There seems to be some correlation among the Pgm-1 alleles and the horticultural groups of pineapple (Table 5.1). All cultivars within the "Cayenne", "Queen", and "Maipure" groups exhibit identical banding patterns, i.e., FM. Phylogenetic relationships between these groups will be discussed in Chapter VI. Most of the cultivars in the "Spanish" group had a common banding pattern for Pgm-1, i.e., SS. However, the 4 cultivars in the "Abacaki" group, showed different allelic combinations for Pgm-1, i.e., FM, MS, SS.

Pineapple cultivars are classified into horticultural groups without regard to their origin or genetic relationships, e.g., 'PR-1-67' is included in the "Spanish" group even though it is a known hybrid (Samuels, 1970; Leal and Soule, 1977). Similarly, other cultivars could be of hybrid origin, which would explain the isozyme banding variability observed between individuals within a group. 'Caicara' is from the same region as 'Panare' and

'Brecheche' and it may have been derived from these. 'Black Jamaica' may be a hybrid between 'Smooth Cayenne' x 'Red Spanish', and 'Valera Amarilla' may be a hybrid of 'Perolera' x 'Red Spanish', since the parents and hybrids are all cultivated in the same regions. 'Cacho de Venado', which has spiny leaves and conical-shape fruit, has been included in the "Abacaki" group created by Py and Tisseau (1965). Based on isozyme analysis of the Pgm-1 locus, it appears to be more related to the "Spanish" group. Furthermore, 'Cacho de Venado' is from the same region as 'Monte Oscuro' and the feral type 086 (Monagas State, Venezuela). The banding pattern of these cultivars also suggests a hybrid origin for 'Cacho de Venado'.

Species Identification

Peroxidase and phosphoglucomutase banding patterns for 7 Ananas species are shown in Table 5.5, Figs. 5.2 and 5.3. Based on the banding patterns of peroxidase and phosphoglucomutase, 5 of the 7 species can be distinguished. The 2 exceptions are A. comosus, which includes all of the commercial pineapple cultivars, and A. monstrosus. Ananas comosus, as indicated in the previous section, exhibited high levels of isozyme polymorphism, particularly for the Pgm-1 locus. Ananas monstrosus also exhibited isozyme polymorphism in the 2 individuals tested.

The amount of polymorphism observed within A. comosus may be attributed to the present very broad circumscription

of this species. Smith (1961) separates A. comosus from the other Ananas species by the length of the fruit, i.e., greater than 15 cm. Thus, in this classification system, this species includes all the edible pineapples regardless of other morphological differences such as leaf color, presence or absence of spines, and fruit morphology. The same flexibility is not allowed in the classification of the other species and only individuals that exactly fit the description, which includes leaf, flower, and fruit morphology and growth habit, are included.

Ananas monstrosus was originally described by Carriere (1870) and included in the later classification by Smith (1961). The description was based on a single individual plant found. It differs from A. comosus (pineapple) only in that the fruit lacks a crown. The validity of this species has been questioned by Antoni (1983), who reported finding a mutation of the cultivar 'Negrita' that mimics the A. montrosus characteristic, i.e., lack of crown. Based on growth habit and leaf and fruit morphology, the 2 plants representing A. monstrosus used in this study appear to be crown mutations of 'Perola' and 'Red Spanish' respectively. The peroxidase and phosphoglucomutase banding patterns observed for these plants are also typical of 'Perola' and 'Red Spanish'. The taxon thus seems to be of polyphyletic origin. The questionable validity of A. monstrosus as a species is further corroborated by the fact that crown

mutations have commonly been seen in other pineapple cultivars by the author.

The lack of intra-specific variability observed in the isozyme banding patterns of A. ananassoides, A. bracteatus var. tricolor, A. lucidus, A. nanus, and A. parguazensis may have been due to the low sample number (2) used to characterize each species. However, when the electrophoretic data from all 5 of these species are considered together, it is quite surprising that low inter-specific variability was detected as well. It may be that this lack of variability reflects the narrow circumscription of these species. Many of the feral types used in this study closely resembled individuals fitting the definition of species recognized by Smith (1961), but they were not included in any of the prsently recognized species because they differed in 1 or 2 characters from the species descriptions given by Smith and Downs (1979). These genotypes may belong within the limits of some of the presently delimited species, thus giving this taxa greater intra-specific variability, as in the case of the different clones of pineapple (A. comosus). Perhaps the most valid explanation for the high degree of intra-specific homology observed in all the 'Ananas' species, except for A. comosus, is that they are self-pollinated. In contrast, A. comosus is self-incompatible, with obligate out-crossing favoring a high degree of isozyme and allelic heterogenity.

Feral Type Identification

The phosphoglucomutase banding patterns for the 29 feral types characterized electrophoretically are shown in Table 5.6, Fig. 5.3. The full range of isozymic polymorphism at each of the 3 phosphoglucomutase loci was represented in this diverse collection of genotypes. The Pgm-1 locus was particularly polymorphic, with all of the 5 alleles represented, i.e., L, S, M, F, and P. Two of these alleles, L and P, were unique to this collection and had not been observed in any of the species or pineapple cultivars previously characterized. Nine different banding patterns (SL, ML, SS, MS, MM, FM, FS, FF, and PS) were observed at the Pgm-1 locus. Five of these (SL, ML, MM, FF, and PS) were new allelic combinations not previously observed.

The FF Pgm-1 genotype was observed in P.I. 095, typical pineapple plants that produce large, conical 3 kg fruit. The 2 sampled plants of this introduction were collected from a small plot of plants being cultivated by the Piaroas Indian River community of El Gavilan. The region is an isolated portion of dense tropical forest located in the north central portion of the Amazonian Territory in southern Venezuela. The cultivated plants were reportedly collected from the wild by the local Indians.

The Pgm-1 genotype MM was observed in the 2 plants of the introduction P.I. 487443. These plants were collected from the tropical rain forest region of San Carlos de Rio Negro. This region is located in the southernmost portion of

Venezuela, on the border with Brazil. Two other introductions, P.I. 487440 and P.I. 487445, were also collected from this location. Their genotypes at the Pgm-1 locus were both heterozygous MS. Both the MM and MS plants from this location were smooth-leafed pineapples with similar growth habits. It appears likely that hybridization between plants with 2 MS alleles gave rise to the MM genotype (Table 5.6).

The Pgm-1 allele L appeared in P.I. 072-1 (ML) and P.I. 085 (SL). These introductions were collected from the southeastern portion of Venezuela. All of the plants have leaves with few spines and bear purple-colored fruit. Based on these morphological characteristics, they would be included in the "Spanish" horticultural group of pineapples.

The Pgm-1 allele P was observed only in P.I. 115. This introduction is particularly interesting. Morphologically, these plants appear intermediate between the primitive A. ananassoides and the domestic A. comosus (Fig. 5.4) The plants were collected in the wild from a large homogenous population of plants growing in an arid sandy region near the Cunavichito River in the Apure State of southwestern Venezuela.

Plant introductions 046, 079, and 094; 097 and 108; and 064 were putatively grouped under A. ananassoides, A. parguazensis, and A. nanus, respectively, because of their phenotypic similarities to these species (Table 5.6). When these introductions were electrophoretically characterized,

the observed banding patterns at the Pgm-1 locus were all identical with those observed in the valid individuals of the species. However, isozyme polymorphisms were recorded in these putative species members at both the Pgm-2 and the Pgm-3 loci. The morphological and electrophoretic similarities of these P.I.s to the genotypes of the respective species appear to indicate that the plants should be considered as members of the species.

Plant introductions 116, 117, 188, 189, 193, and 25291 were included in the group "Others" (Table 5.6) because they exhibited little morphological similarity to any of the presently delimited Ananas species. All of these genotypes, with the exception of P.I. 193, produce fruit less than 15 cm long and therefore would not be considered true pineapples. Plant introduction 193 produces fruit more than 15 cm long and has leaves with retrorse spines similar to those of A. parguazensis. Plant introduction 189 produces an 11 cm edible fruit with a Brix of 16.5. This diverse collection of genotypes showed considerable polymorphism for the 3 phosphoglucomutase loci analyzed.

Conclusions

1. Electrophoretic isozyme determination of peroxidase and phosphoglucomutase allows the unique identification of 15 of the 27 pineapple cultivars examined. No distinction could be made between a cultivar and its sport.

2. The hybrid origin of 'PR-1-67' from a cross of 'Red Spanish' x 'Smooth Cayenne' was confirmed by their isozyme banding patterns.
3. The hyphothesis that some cultivars originated as sports was tested. The isozyme data comfirms the sport origin of 'Esmeralda' and 'Dupuis Smooth' from 'Smooth Cayenne', and 'Cumanesa' from 'Red Spanish'. Conversely, 'Bumanguesa' does not appear to be a sport of 'Perolera'.
4. The isozyme banding pattern 'Panare', and its morphological resemblance to the "Queen" group, indicates the presence of locally-originated "Queen" types growing in the Western Hemisphere. The origin of "Queen" pineapples from a 'Panare'-like wild type is suggested.
5. A correlation between the Pgm-1 alleles and the horticultural groups of pineapple was found. Based on this fact and on plant locations, a hybrid origin for 'Caicara', 'Black Jamaica', 'Valera Amarilla', and 'Cacho de Venado' is indicated.
6. Based of banding patterns of peroxidase and phosphoglucomutase, 5 of the 7 species could be distinguished. The 2 exceptions were A. comosus, which showed high polymorphism in its cultivars, and A. monstrosus.
7. The A. monstrosus plants were confirmed to be mutations of pineapple cultivars based on isozyme

patterns. The questionable validity of this species is corroborated.

8. The lack of intra-specific variability observed in isozyme banding patterns of Ananas species, except A. comosus, is maybe due to the present restrictive delimitation of these species, and/or to the self-compatible character of these species compared to the self-incompatible A. comosus.
9. Increased polymorphism for the Pgm-1 locus was observed in the feral types. This variability was represented either by 2 different alleles or new allelic combinations.
10. Plant introductions putatively grouped under species with similar morphology also showed similarity in their isozyme banding patterns.

Table 5.1. Peroxidase and phosphoglucomutase isozyme banding patterns observed for pineapple cultivars.

Hort.[z] group	Cultivar	Isozyme genotypes[y]				
		Per-2	Per-3	Pgm-1	Pgm-2	Pgm-3
Abacaki	Abaka	FF	FF	MS	FS	SS
	Cacho de Venado	FS	FS	SS	FF	SS
	Injerta	FS?	FS	MS	FS	SS
	Perola	FF	FS	FM	FF	SS
Cayenne	Dupuis Smooth	FF	FF	FM	FF	SS
	Esmeralda	FF	FF	FM	FF	SS
	Smooth Cayenne	FF	FF	FM	FF	SS
Queen	Queen	FS	SS	FM	SS	SS
	Panare	FS	SS	FM	SS	SS
Maipure	Bumanguesa	FF	FF	MS	FS	FS
	Maipure	SS	SS	MS	FS	SS
	Perolera	FS	FS	MS	FS	SS
	Pina de Brazil	FS	FF	MS	FS	FS
	Rondon	FS	FS?	MS	FF	FF
Spanish	Black Jamaica	FF	FF	FS	FS	SS
	Cabezona	FF	FF	SS	FS	SS
	Caicara	FF	FS?	MS	FS	FS
	Cumanesa	FF	FF	SS	SS	SS
	Monte Oscuro	FF	FF	SS	FS	FS
	Nacional	FF	FF	SS	SS	SS
	Negrita	FF	SS?	SS	SS	SS
	PR-1-67	FF	FF	MS	FS	SS
	Red Spanish	FF	FF	SS	SS	SS
	Valera Amarilla	FF	FS	MS	SS	SS
Others	Brecheche	FF	FS	SS	FS	FS
	Cambray	FS	FS	SS	FS	FS
	Masmerah	FF	FS	SS	SS	SS

[z]Groups are based on presence or absence of spines and fruit morphology.

[y]"?" indicates a doubtful determination.

Table 5.2. Pineapple cultivar classification based on peroxidase banding patterns.

Per-2/Per-3 genotype	Pattern[z]	Cultivar
FF/FF	A_1	Dupuis Smooth, Esmeralda, Monte Oscuro, National, PR-1-67, Red Spanish, Smooth Cayenne
FF/FS	A_2	Brecheche, Caicara, Masmerah, Perola, Valera Amarrilla
FF/SS	A_3	Negrita (?)[y]
FS/FF	A_4	Pina de Brazil
FS/FS	A_5	Cacho de Venado, Cambray, Injerta, Perolera, Rondon
FS/SS	A_6	Panare, Queen
SS/SS	A_7	Maipure

[z]Patterns are shown in Fig. 5.1.

[y]"?" indicates a doubtful classification.

Table 5.3. Pineapple cultivar classification based on phosphoglucomutase banding patterns.

Pgm-1/Pgm-2/ Pgm-3	Pattern[z]	Cultivar
FM/FF/SS	B_1	Dupuis Smooth, Esmeralda, Perola, Smooth Cayenne
FM/SS/SS	B_2	Queen, Panare
FS/FS/SS	B_3	Black Jamaica
MS/FF/FF	B_4	Rondon
MS/FS/FS	B_5	Bumanguesa, Caicara, Pina de Brazil
MS/FS/SS	B_6	Abaka, Injerta, Maipure, Perolera, PR-1-67
MS/SS/SS	B_7	Valera Amarilla
SS/FF/SS	B_8	Cacho de Venado
SS/FS/SS	B_9	Brecheche, Cambray, Monte Oscuro
SS/FS/SS	B_{10}	Cabezona
SS/SS/SS	B_{11}	Cumanesa, Masmerah, Nacional, Negrita, Red Spanish

[z]Patterns are shown in Fig. 5.1.

Table 5.4. Pineapple cultivar identification based on peroxidase (Per) and phosphoglucomutase (Pgm) isozyme banding patterns.

Class	Per	Pgm	Cultivar
1	A_1^1	B_1	Dupuis Smooth, Esmeralda, Smooth Cayenne
2	A_1^1	B_3	Black Jamaica
3	A_1^1	B_5	Bumanguesa
4	A_1^1	B_6	Abaka, PR-1-67
5	A_1^1	B_9	Monte Oscuro
6	A_1^1	B_{10}	Cabezona
7	A_1^1	B_{11}	Cumanesa, Nacional, Red Spanish
8	A_2^2	B_2	Perola
9	A_2^2	B_5	Caicara
10	A_2^2	B_7	Valera Amarilla
11	A_2^2	B_9	Brecheche
12	A_2^2	B_{11}	Masmerah
13	A_3^2	B_{11}	Negrita (?)[z]
14	A_4^3	B_5	Pina de Brazil
15	A_5^4	B_4	Rondon
16	A_5^5	B_6	Injerta, Perolera
17	A_5^5	B_8	Cacho de Venado
18	A_5^5	B_9	Cambray
19	A_6^6	B_2	Panare, Queen
20	A_7^7	B_6	Maipure

[z]"?" indicates a doubtful classification.

Table 5.5. Peroxidase and phosphoglucomutase isozyme banding patterns observed for *Ananas* species.

Species	Isozyme genotypes[z]				
	Per-2	Per-3	Pgm-1	Pgm-2	Pgm-3
A. ananassoides	FF	FS	MS	SS	FS?
A. bracteatus var. *tricolor*	FS?	FF	MS	SS	SS
A. comosus[y]	FF FS SS	FF FS SS	FM FS MS SS	FF FS SS	FF FS SS
A. lucidus	FF	SS	SS	SS	SS
A. monstrosus[x]	FF FF	FS FF	FM SS	FF SS	SS SS
A. nanus	SS	FF?	SS	SS?	SS?
A. parguazensis	--	--	SS	FS?	SS

[z] "?" indicates a doubtful determination; "-" indicates data not available.

[y] Includes pineapple cultivars.

[x] Plants from 2 cultivars that resemble this species were analyzed.

Table 5.6. Phosphoglucomutase isozyme banding patterns observed for various Ananas genotypes.

Species and/or Hort. group	P.I.[z]	Isozyme genotypes[y] Pgm-1	Pgm-2	Pgm-3
A. comosus				
Abacaki	095	FF	FS?	SS
	192	MS	FF	FF
Maipure	092-1	FS	FF	SS
	093	FM	FS?	SS
	126	MS	FF	FF
	196	MS	FS	FS
	487439	MS	FS	FS
	487440	MS	FS	FS
	487443	MM	FS	SS
	487445	MS	FS	FS
Spanish	072-1	ML	FS	SS
	085	SL	FF	FF
	086	MS	FF	FS
	092-2	SS	FS	SS
	110	FS	FS?	FS?
	487442	MS	SS	SS
A. ananassoides	046	MS	SS?	FS
	079	MS	SS	SS
	094	MS	SS	FF
A. parguazensis	097	SS	FS	FS
	108	SS	FF	SS
A. nanus	064	SS	SS?	SS
Others[x]	115	PS	SS?	FF?
	116	SS	FS?	SS
	117	SS	FS	FS
	188	MS	FS	SS
	189	SS	FS	FF
	193	FS	FF	FF?
	27285	MS	SS	SS

[z]Plant introduction numbers.

[y]"?" indicates a doubtful determination.

[x]Botanical characteristics of the plant do not fit any of the presently described species.

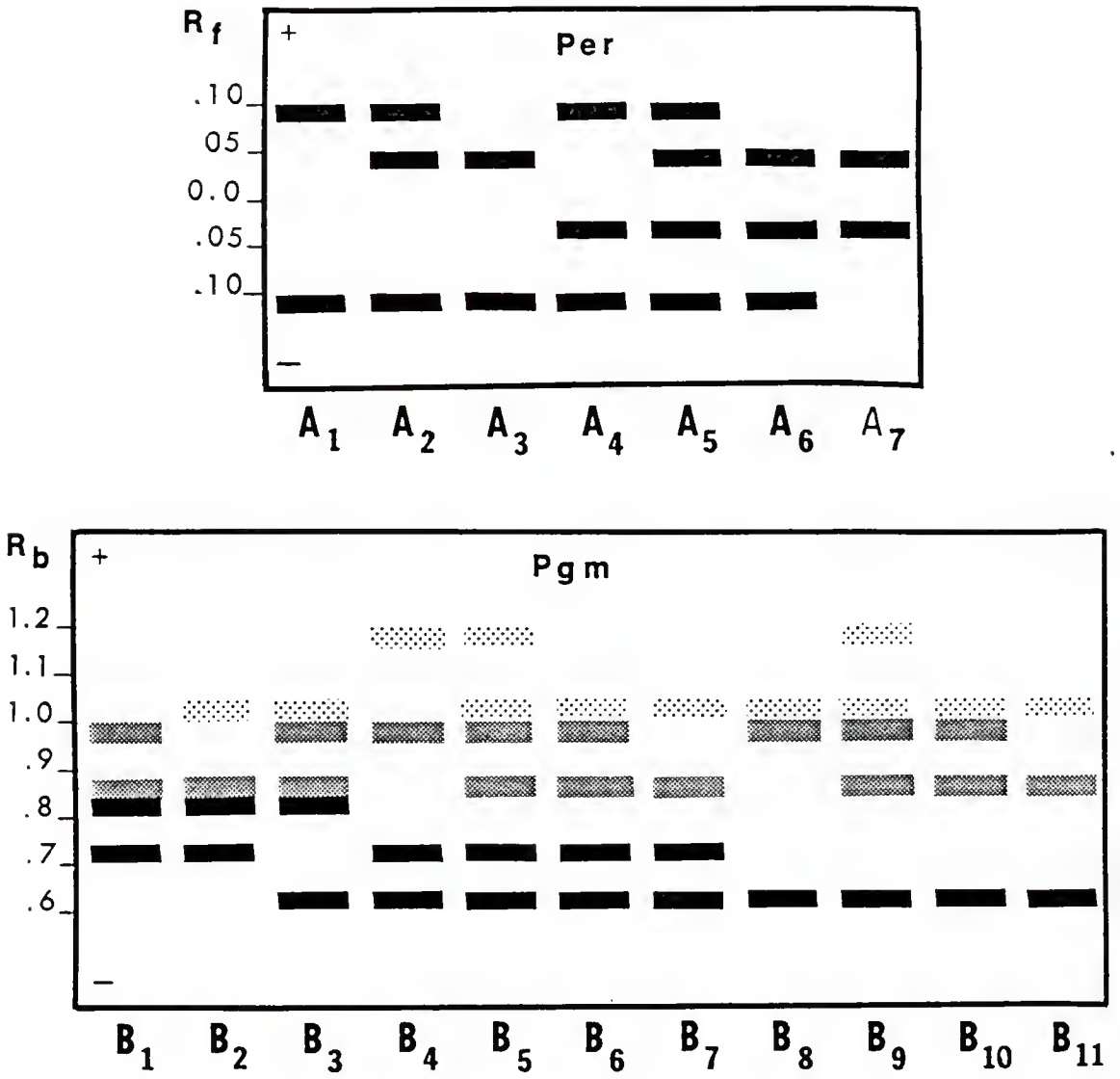


Figure 5.1. Schematic representation of peroxidase (Per) and phosphoglucomutase (Pgm) isozymes in pineapple cultivars.

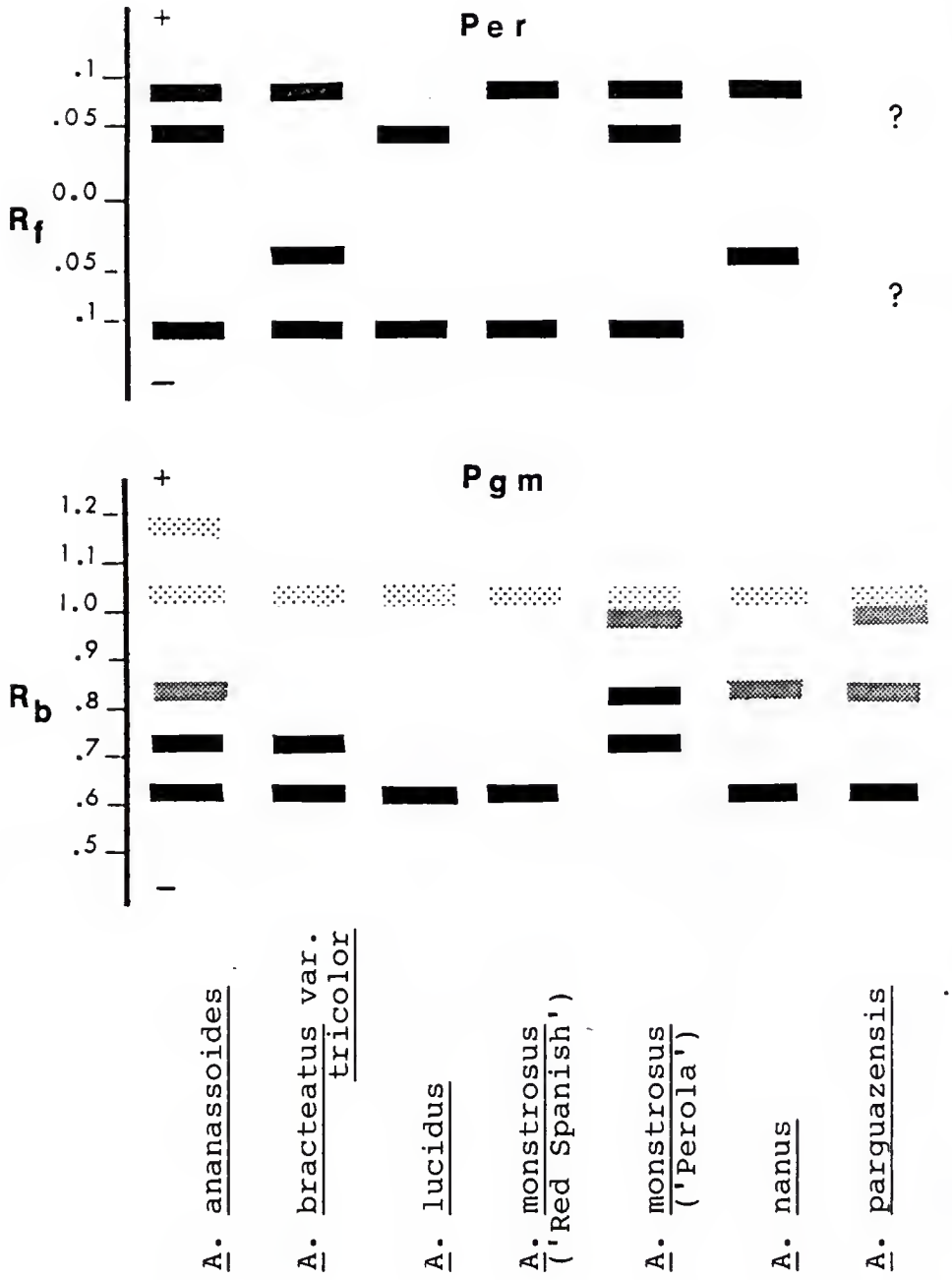
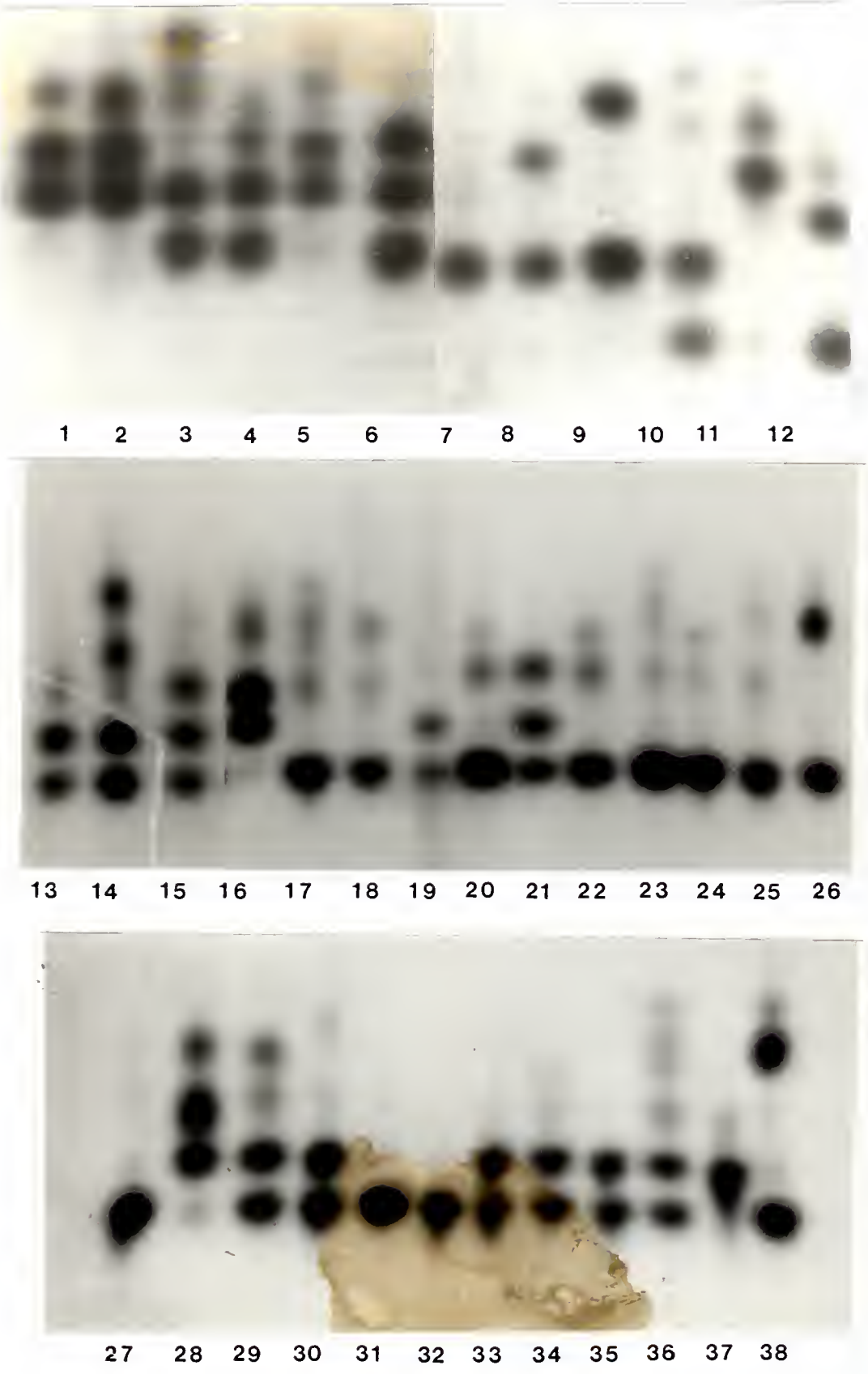


Figure 5.2. Schematic representation of peroxidase (Per) and phosphoglucomutase (Pgm) isozymes in Ananas species. Ananas comosus is represented by the cultivars depicted in Fig. 5.1.

Figure 5.3. Variation in phosphoglucomutase isozyme in Ananas genotypes leaf extracts. The sequence is: 1. 'Smooth Cayenne'; 2. 'Perola'; 3. 'Caicara'; 4. 'Perolera'; 5 and 16. 'Panare'; 6. 'Bumanguesa'; 7. 'Cambray'; 8. P.I. 193; 9, 26 and 38. P.I. 115; 10. P.I. 085; 11. A. monstrosus ('Perola'?); 12. P.I. 072-1; 13. P.I. 487442; 14. 'Rondon'; 15. A. bracteatus var. tricolor; 17, 24 and 25. 'Brecheche'; 18. A. parguazensis; 19. P.I. 196; 20. A. lucidus; 21. 'Injerta'; 22. 'Cacho de Venado'; 23. 'Monte Oscuro'; 27. 'Cabezona'; 28. 'Queen'; 29. P.I. 25291; 30. P.I. 487445; 31. 'Masmerah'; 32. P.I. 097; 33 and 36 'Pina de Brazil'; 34. 'Abaka'; 35. P.I. 487440; 37. P.I. 487443. Sample origin is at the bottom of each photograph and band migration is anodal.


1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38

CHAPTER VI
PHENETIC RELATIONSHIPS IN *ANANAS* BASED ON ISOZYME ELECTROPHORETIC ANALYSIS

Introduction

Phenetic studies are particularly valuable for the efficient utilization and preservation of germplasm sources. When a genus has been well characterized, germplasm collections can be made that reflect the natural diversity of the crop. Plant breeders can quickly locate genotypes that possess those alleles most useful in hybridization schemes. One way in which electrophoretic isozyme analysis has been used successfully is in the assessment of the genetic similarities between populations (Johnson et al., 1967; Sheen, 1970; Smith et al., 1970; Bassiri, 1977; Loukas and Pontikis, 1979; Meerow, 1986; Panda et al., 1986). In general, close relationships are expected in isozyme banding patterns between 2 or more populations which have common parentage (Bassiri and Adams, 1978).

Isozymic studies from Chapter IV and V were utilized to define phenetic relationships between populations of cultivated pineapples. Special emphasis was placed on

comparing the commercial cultivars with the genotypes collected from the center of origin.

The determination of how many individuals and populations to sample before a confident statement can be made regarding the amount of genetic divergence between taxa is an important consideration in the use of electrophoretical evidence (Gottlieb, 1977). Marshall and Brown (1975) showed that, in the extreme case of 20 alleles at a locus, with frequencies of 0.05 each, a random sample of 60 individuals will include, with 95% confidence, 1 copy of each allele. Thus, the sampling number used in this study (62 genotypes) should include all possible alleles at the loci analyzed.

A number of statistical coefficients have been devised to place allelic frequency data into a single statistical value (Cavalli-Sforza and Edwards, 1967; Hendrick, 1971). Two such values or statistics are genetic similarity and genetic distance (Gottlieb, 1977). Small sample size and low number of loci are 2 variables which decrease the precision of the genetic distances estimation (Nei, 1978). Unfortunately, the limited number of individuals per genotype (2) and number of loci (7) analyzed in this study do not allow any statistically significant explorations of genetic variation among the genotypes. Another approach for estimating the genetic distances of conspecific populations has been suggested by Gottlieb (1975). This method makes use

of the presence or absence of alleles rather than their frequencies and was used in this study.

Material and Methods

A phenetic analysis of 62 Ananas genotypes was conducted using the genetic isozyme models of Chapter IV and isozyme polymorphism data of Chapter V. The analysis consisted of dendographic representations of phosphoglucomutase and peroxidase isozyme polymorphisms.

The genotypes were numbered from 1-62 as shown in Table 6.1. Alleles from Pgm-1, Pgm-2, Pgm-3, Per-2, and Per-3 loci were enumerated according to the following system: Pgm-1 L=1, S=2, M=3, F=4, P=5; Pgm-2 F=6, S=7; Pgm-3 F=8, S=9; Per-2 F=10, S=11; Per-3 F=12, S=13. All of the cultivars were characterized as to the presence or absence of each of the 13 alleles (Table 6.2). The feral types and species were analyzed using only the 9 alleles of phosphoglucomutase. From this information, 3 data sets were constructed. One data set included the cultivars using both phosphoglucomutase and peroxidase data. A second data set was constructed with the feral types and species using only phosphoglucomutase data and a third grouping combined the information from these 2 data sets.

Distance coefficients were calculated based on the unweighted pair method of Sneath and Sokal (1973), using a computer program and the data from Table 6.2. The results are presented as cluster analysis dendograms. Individual

genotypes that were found clustered together in the dendograms are then compared for morphological similarities.

Results and Discussion

The cluster analysis dendogram of the 27 pineapple cultivars, based on genetic distance coefficients of the isozyme banding patterns, is shown in Fig. 6.1. Cultivars that cluster together at a distance coefficient (DC) of 0.0 are assumed to possess nearly identical banding patterns. In general, the cultivars from a common horticultural group (Table 5.2) did not exhibit close phenetic relationships as determined by cluster dendograms and the DCs when the isozyme banding patterns of both phosphoglucomutase and peroxidase were considered together. Frequently, cultivars possessing distinctly different growth habits, leaf morphologies (including spines), and fruit morphologies exhibited close isozymic relationships.

The isozyme relationships among the feral types and species of Ananas are illustrated in the dendogram shown in Fig. 6.2. As in the previous dendogram few morphological similarities could be distinguished among the various individuals or groups of individuals which exhibited close isozymic relationships.

The dendogram in Fig. 6.3 considers the isozymic relationships of all the genotypes together. Included are the Ananas species, the feral types, and the major pineapple cultivars. The Ananas species do not cluster at close DC

coefficients, except for A. nanus (61) and A. lucidus (58) which cluster at a DC of 0.0.

Most of the cultivars fell into one of several discrete clusters at a DC of 0.0, indicating a close relationship between the members of a cluster. This type of clustering was not observed in the feral types. 'Monte Oscuro' (19), an isolated small cultivar grown in eastern Venezuela and characterized by large plants (>2 m) and fruits (>10 kg) was an exception.

One of the most interesting clusters is formed by a branch at a DC of 0.204 (indicated by the asterisk in Fig. 6.3). This cluster includes some of the most economically important pineapple cultivars, including 'Smooth Cayenne' (6) and its sports 'Esmeralda' (5) and 'Dupuis Smooth' (4) and 'Perola' (9) and a 'Perola' crown mutation (60). Slightly less related members of this "Smooth Cayenne" group include P.I. 093 (32), 'Queen' (7), 'Panare (8), P.I. 48743 (36) and, P.I. 072-1 (38). The relationships contained within this cluster may provide some evidence on the origin of 'Smooth Cayenne'. It has been demostrated in Drosophila (Duke and Glassman, 1968) and in rice (Siddiq et al., 1972) that the electrophoretic mobility of isozymes tends to be reduced as the populations become more evolutionarily advanced. All the genotypes forming the "Smooth Cayenne" cluster possess the fast mobility isozymes (Tables 5.2 and 5.7), thus suggesting that they may be more ancient than other cultivated pineapples.

'Smooth Cayenne' and its sports are by far the most important cultivars of pineapple today. Collins (1960) has traced the early history of 'Smooth Cayenne' back to 5 plants sent by Perrottet from French Guiana to France sometime before 1840. In France, they were vegetatively propagated and shipped to England, and then to Australia, Florida, Jamaica, and Hawaii. Collins (1960) suggests that, because Perrottet used the name "mai-pouri" for his pineapple, the original plants may have been obtained from the Maipure Indians of the upper Orinoco River in south Venezuela. All the other genotypes forming the cluster with 'Smooth Cayenne' come from this geographic region. 'Panare' is grown in small areas close to the margin of the Orinoco River in Bolivar State, Venezuela. 'Queen' seems to have originated from 'Panare', as discussed in Chapter V. 'Perola' is grown in northern Brazil, and its origin has not been established, but it presumably comes from the northern Amazonian region. Plant introductions 095, 477443, and 072-1 were collected in the Amazonian Territory of southern Venezuela. The fact that 'Smooth Cayenne' clusters with these types instead of with any other cultivar suggests that, as Collins believed, it may have been collected in the basin of the Orinoco River and perpetuated vegetatively, so that it still resembles the feral types growing in the region.

The rest of the cultivated varieties of pineapple do not possess the fast mobility bands observed in the "Smooth

Cayenne" cluster. This fact sugggests that these cultivars probably had a different origin than 'Smooth Cayenne'. Collins (1960) speculates that many cultivars may have originated as hybrids in greenhouses in Europe, where pineapple was very popular in the 19th century.

The feral genotype P.I. 115 (51) exhibits a particularly interesting and unique isozyme banding pattern and remains a distant outlayer from all other types, joining them at a DC of 0.495 (Fig. 6.3). It possesses the fastest mobility allozyme band (P) at the Pgm-1 locus, suggesting that it may be the most primitive of the genotypes tested. Gottlieb (1977) found a high degree of genetic similarity between conspecific populations. He suggested that if a population is discovered which has novel alleles, this is strong evidence that the population should be a distinct taxon. Thus, P.I. 115 should be further examined with this in mind, as it may represent an important turning point in the evolution of domestic pineapples.

A number of genotypes, i.e., P.I.s 072-1 (38), 085 (39), 086 (40), 093 (32), 095 (26), 189 (55), 193 (43), and 487443 (36) appear to be rather isolated individuals. These genotypes join clusters with other genotypes at DCs greater than 0.18. The only common character of these feral types is the fact that they were all collected from southern Venezuela; otherwise they greatly differ in their morphological characteristics. This indicates the broad genetic diversity that exists between feral types and the

importance of continuing to collect types from this region, as it seems indeed to be the center of origin of the Ananas species.

Table 6.1. Designation of genotypes analyzed electrophoretically.

ID no.	Genotype
1	Abaka
2	Cacho de Venado
3	Injerta
4	Dupuis Smooth
5	Esmeralda
6	Smooth Cayenne
7	Queen
8	Panare
9	Perola
10	Bumanguesa
11	Maipure
12	Perolera
13	Pina de Brazil
14	Rondon
15	Blanca
16	Cabezona
17	Caicara
18	Cumanesa
19	Monte Oscuro
20	Nacional
21	Negrita
22	PR-1-67
23	Red Spanish
24	Valera Amarilla
25	Brecheche
26	Cambray
27	Masmerah
28	P.I. 095
29	P.I. 192
30	P.I. 126
31	P.I. 092-1
32	P.I. 093
33	P.I. 196
34	P.I. 487439
35	P.I. 487440

Table 6.1. continued.

ID no.	Genotype
36	P.I. 487443
37	P.I. 487445
38	P.I. 072-1
39	P.I. 085
40	P.I. 086
41	P.I. 092-2
42	P.I. 110
43	P.I. 193
44	P.I. 27285
45	P.I. 046
46	P.I. 079
47	P.I. 094
48	P.I. 064
49	P.I. 097
50	P.I. 108
51	P.I. 115
52	P.I. 116
53	P.I. 117
54	P.I. 188
55	P.I. 189
56	A. ananassoides
57	A. bracteatus
58	A. lucidus
59	A. monstrosus (Red Spanish)
60	A. monstrosus (Perola)
61	A. nanus
62	A. parguazensis

Table 6.2. Presence-absence data matrix for electrophoretic analysis of Ananas genotypes.

ID no.[y]	Band number[z]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0	1	1	0	0	1	1	0	1	1	0	1	0
2	0	1	0	0	0	1	0	0	1	1	1	1	1
3	0	1	1	0	0	1	1	0	1	1	1	1	1
4	0	0	1	1	0	1	0	0	1	1	0	1	0
5	0	0	1	1	0	1	0	0	1	1	0	1	0
6	0	0	1	1	0	1	0	0	1	1	0	1	0
7	0	0	1	1	0	0	1	0	1	1	1	0	1
8	0	0	1	1	0	0	1	0	1	1	1	0	1
9	0	0	1	1	0	1	0	0	1	1	0	1	1
10	0	1	1	0	0	1	1	1	1	1	0	1	0
11	0	1	1	0	0	1	1	0	1	0	1	0	1
12	0	1	1	0	0	1	1	0	1	1	1	1	1
13	0	1	1	0	0	1	1	1	1	1	1	1	0
14	0	1	1	0	0	1	0	1	0	1	1	1	1
15	0	1	0	1	0	1	0	0	1	1	0	1	0
16	0	1	0	0	0	1	0	0	1	1	0	1	0
17	0	1	1	0	0	1	1	1	1	1	0	1	1
18	0	1	0	0	0	0	1	0	1	1	0	1	0
19	0	1	0	0	0	1	0	1	0	1	0	1	0
20	0	1	0	0	0	0	1	0	1	1	0	1	0
21	0	1	0	0	0	0	1	0	1	1	0	0	1
22	0	1	1	0	0	1	1	0	1	1	0	1	0
23	0	1	0	0	0	0	1	0	1	1	0	1	0
24	0	1	1	0	0	0	1	0	1	1	0	1	1
25	0	1	0	0	0	1	1	1	1	1	0	1	1
26	0	1	0	0	0	1	1	1	1	1	1	1	1
27	0	1	0	0	0	0	1	0	1	1	0	1	1
28	0	0	0	1	0	1	1	0	1				
29	0	1	1	0	0	1	0	1	0				
30	0	1	1	0	0	1	0	1	0				
31	0	1	0	1	0	1	0	0	1				
32	0	0	1	1	0	1	1	0	1				
33	0	1	1	0	0	1	1	1	1				
34	0	1	1	0	0	1	1	1	1				
35	0	1	1	0	0	1	1	1	1				

Table 6.2. continued.

	Band number[z]												
ID no.[y]	1	2	3	4	5	6	7	8	9	10	11	12	13
36	0	0	1	0	0	1	1	0	1				
37	0	1	1	0	0	1	1	1	1				
38	1	0	1	0	0	1	1	0	1				
39	1	1	0	0	0	1	0	1	0				
40	0	1	1	0	0	1	0	1	1				
41	0	1	0	0	0	1	1	0	1				
42	0	1	0	1	0	1	1	1	1				
43	0	1	0	1	0	1	0	1	0				
44	0	1	1	0	0	0	1	0	1				
45	0	1	1	0	0	0	1	1	1				
46	0	1	1	0	0	0	1	0	1				
47	0	1	1	0	0	0	1	1	0				
48	0	1	0	0	0	0	1	0	1				
49	0	1	0	0	0	1	1	1	1				
50	0	1	0	0	0	1	0	0	1				
51	0	1	0	0	1	0	1	1	0				
52	0	1	0	0	0	1	1	0	1				
53	0	1	0	0	0	1	1	1	1				
54	0	1	1	0	0	1	1	0	1				
55	0	1	0	0	0	1	1	1	0				
56	0	1	1	0	0	0	1	1	1				
57	0	1	1	0	0	0	1	0	1				
58	0	1	0	0	0	0	1	0	1				
59	0	1	0	0	0	0	1	0	1				
60	0	0	1	1	0	1	0	0	1				
61	0	1	0	0	0	0	1	0	1				
62	0	1	0	0	0	1	1	0	1				

[z]Blank spaces indicate data not included in the study.

[y]Refer to Table 6.1 for population designations.

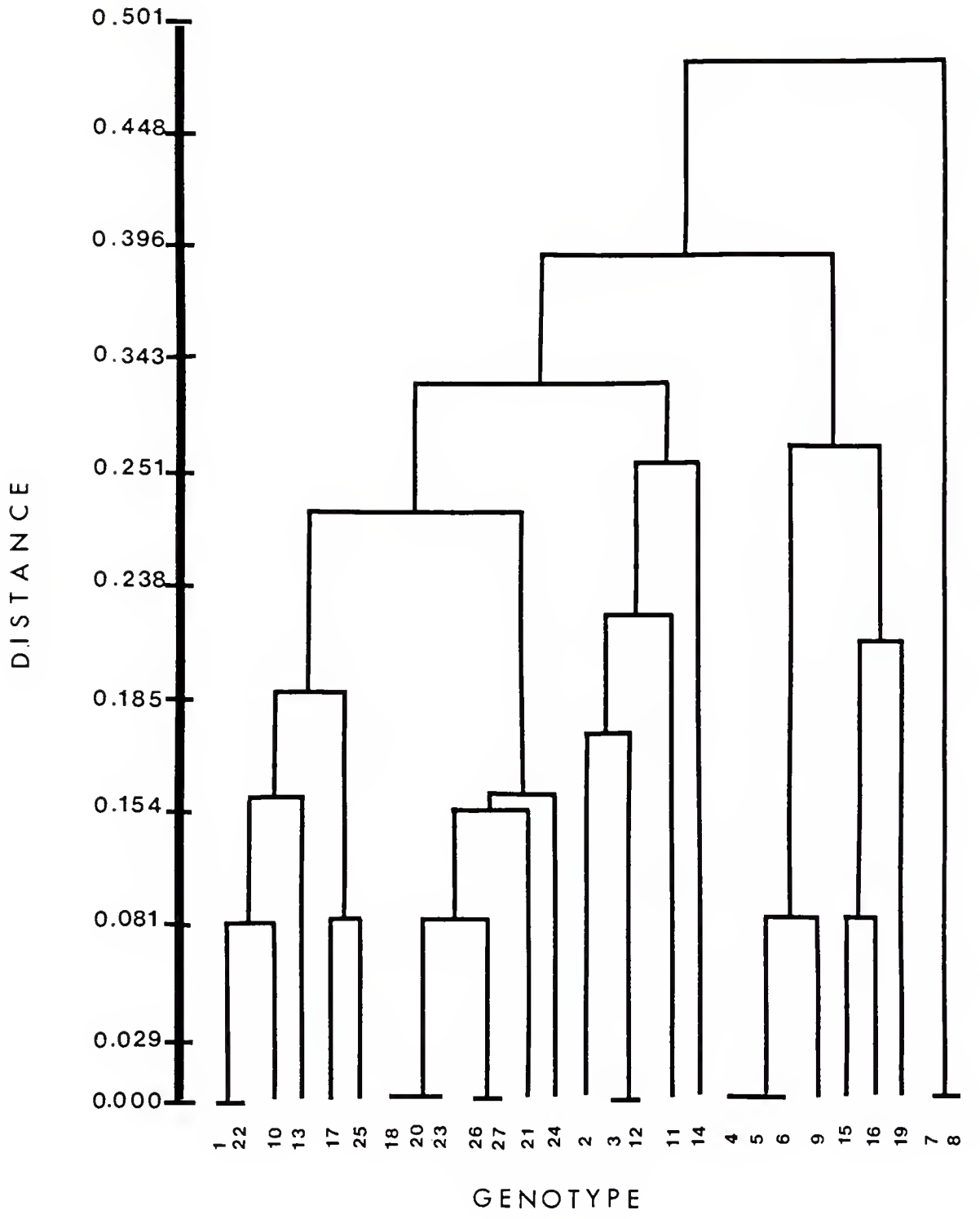


Figure 6.1. Cluster analysis dendogram of 27 pineapple cultivar isozyme phenotypes. Refer to Table 6.1 for genotype designations.

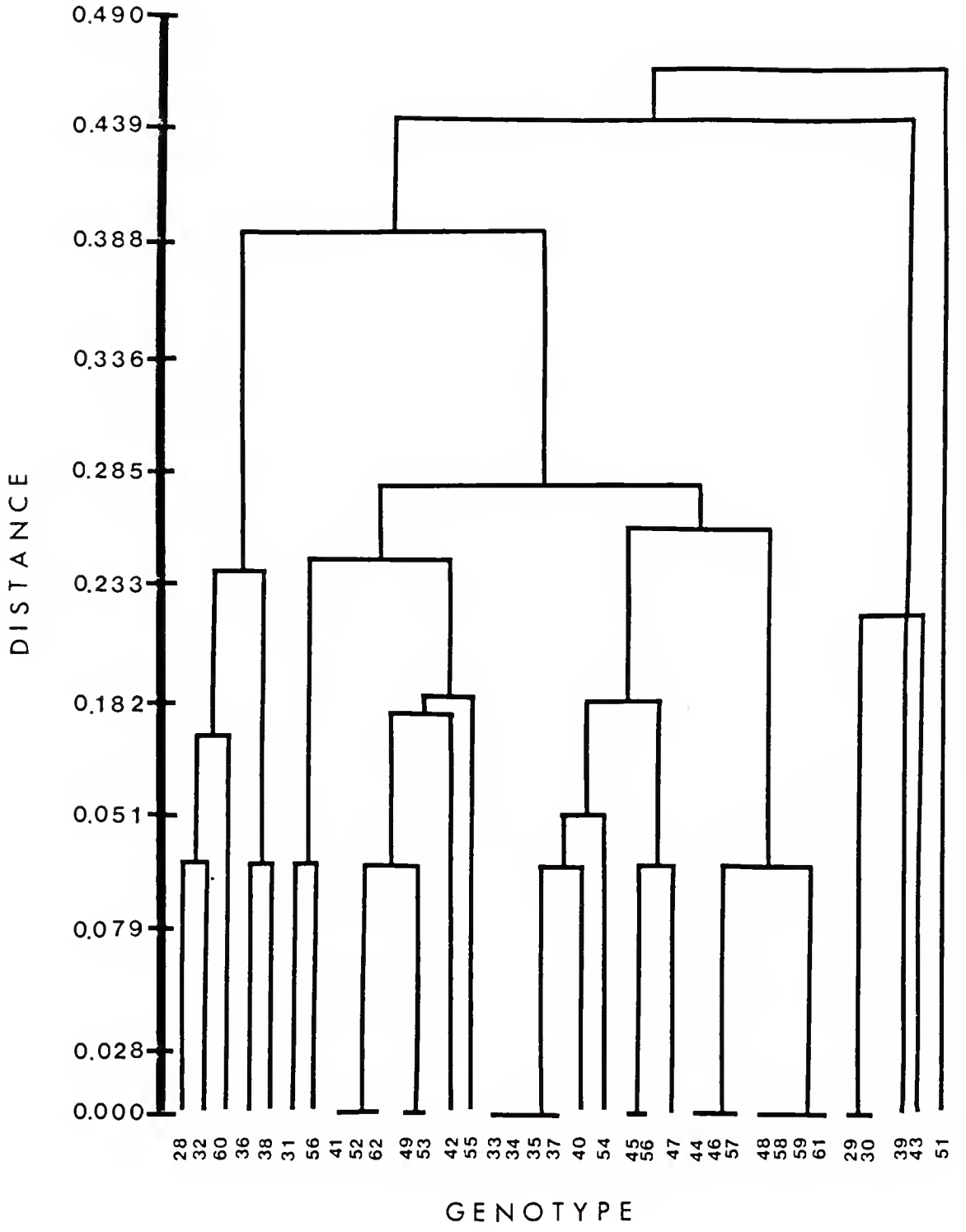


Figure 6.2. Cluster analysis dendogram of isozyme phenotypes of Ananas species and feral types. Refer to Table 6.1 for genotype designation.

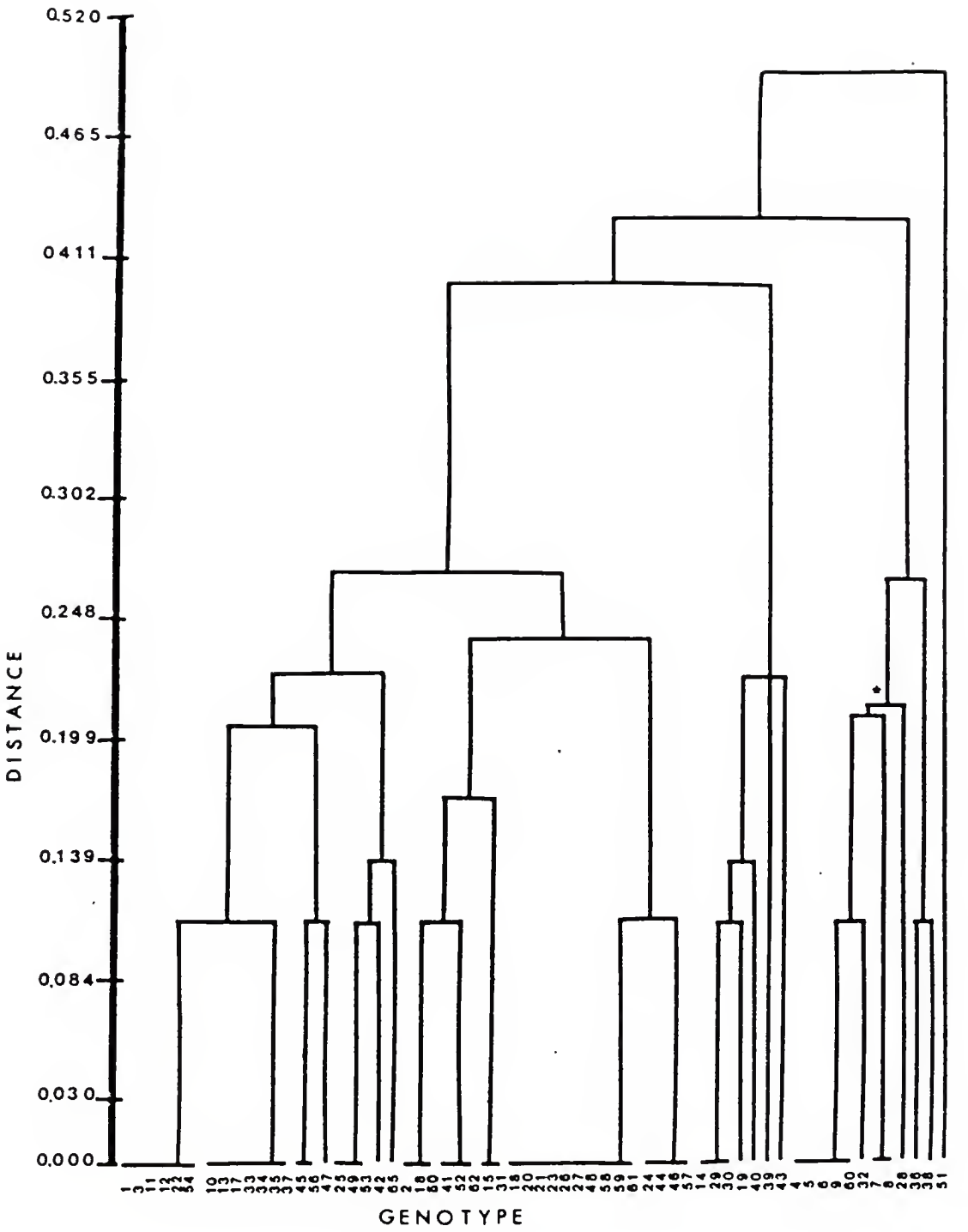


Figure 6.3. Cluster analysis dendogram of the isozyme phenotypes of 62 Ananas genotypes. Refer to Table 6.1 for genotype designations.

CHAPTER VII
CHARACTERIZATION OF REGENERATED PINEAPPLE PLANTS

Introduction

The usefulness of a pineapple plant propagation system is judged by its ability to efficiently establish large numbers of plants in the greenhouse or field and by the clonal fidelity of these plants. A tissue culture multiplication system, as discussed in Chapter III, has been shown to meet this first requirement. The requirement of clonal fidelity will be addressed in this Chapter.

The reports of somaclonal variation in numerous plant species and pineapple in particular, were reviewed in Chapter II. The main purpose of the research reported in this Chapter was to determine if the large amount of variation reportedly found in 'Smooth Cayenne'-regenerated plants (Wakasa, 1979), is a common phenomenon in pineapple cultivars. If other cultivars exhibit less somaclonal variation, then tissue culture may be an effective method for mass propagation of those cultivars. Conversely, if high levels of somaclonal variation are an inherent part of in vitro pineapple propagation systems, then tissue culture may be a useful method of cultivar improvement and development.

Of particular interest is the possibility of obtaining spineless mutants from high quality spiny cultivars such as 'PR-1-67'.

To investigate somaclonal variation, regenerated pineapple plants from axillary bud cultures of 'Perolera', 'PR-1-67', and A. bracteatus var. tricolor were evaluated for variation in both morphological and biochemical characters.

Materials and Methods

Axillary bud cultures were established from a stem of 'Perolera', a slip of A. bracteatus var. tricolor and a crown and a slip of 'PR-1-67', according to the methods described in Chapter III. Regenerated plants from these cultures were used in this study. Plants were grown in 12-cm pots containing a soil mixture composed of a (2:1:1) commercial potting soil, sand, and peat moss. Plants were maintained in a greenhouse under standard practices of irrigation, fertilization, and pest control.

The vegetative characteristics of the regenerated plants, particularly leaf morphology, were observed over a period of 1 year to determine the phenotypic stability of these traits. Electrophoretical evaluation of the regenerates was done for 2 isozyme loci of peroxidase and 3 loci of phosphoglucomutase, following procedures described in Chapter IV.

Results and Discussion

Electrophoretic Characterization

A total of 767 regenerated plants were evaluated for each of the 2 isozyme staining systems (Table 7.1). All of the regenerates, with 2 possible exceptions, exhibited identical isozyme banding patterns for each of the enzyme systems. Two 'PR-1-67' plants gave zymograms with a unique band corresponding to the Per-2 allele S ('PR-1-67' normally had a Per-2 FF genotype). An extra faint band also appeared on the phosphoglucomutase zymograms of these 2 plants. These aberrant zymograms may have been artifacts, because several repeat runs using different leaf samples taken at variuos times produced inconsistent banding patterns. No plants were found that consistently produced a unique banding pattern. There have been other reports in the literature of somaclonal isozyme variation. A peroxidase variant was found by Vardi (1977) among plants regenerated from Citrus protoplasts but studies by Gmitter (1985) and Miller (1986) on a large number of regenerated Citrus plants uncovered no variant zymograms. Menendez et al. (1986) found variant peroxidase zymograms in apple plants regenerated from shoot tip cultures. The enviromental variability of peroxidase is well documented (Hart and Bathia, 1967). Perhaps the variability in peroxidase observed in pineapple was due to developmental or environmental factors rather than genetic changes.

Morphological Characterization

A total of 1900 regenerated plants were evaluated for morphological variation (Table 7.1). High levels of phenotypic variation were observed for a number of diverse vegetative characteristics in the regenerated plants shortly after they were established in soil. Initially the leaf variation included the presence or absence of spines, differences in the amount of surface wax, albino stripes, and the presence of recurved or narrow leaves. Nearly all of the regenerated plants had smooth leaves at the time plants were transferred to soil. As new leaves were formed, however, leaves characteristic of the genotype appeared, i.e., spiny ('PR-1-67' and A. bracteatus var. tricolor) or piping ('Perolera'). During growth in the greenhouse, a large number of plants from the spiny-leaved genotypes exhibited sectors along the leaf edges that were smooth. Two 'PR-1-67' plants with narrow leaves were observed (Fig. 7.1A). These plants died 3 months after being transferred to the soil. Another common aberration in the 'Perolera' regenerated plants was the presence of recurved leaves. This character was transient and, with time, new leaves produced on the plant appeared normal. Four plants, 2 of 'PR-1-67' and 2 of 'Perolera', developed leaves with 1-2 albino stripes (Fig. 7.1B). These plants have continued to express this aberrant phenotype after 1 year of growth in the greenhouse.

No correlation was found in the regenerated plants between observed variability and the explant source. Plants regenerated from cultures derived from crowns, slips, or stems all exhibited similar levels of variation (less than 0.2%). This level of variability was significantly less than that reported by Wakasa (1979) for 'Smooth Cayenne' regenerated plants (34% for lateral buds of crowns and slips). However, the epigenetic or transient variation observed in the regenerated plants shortly after establishment in the soil was more in line with the levels reported by Wakasa (1979). This indicates that the length of time during which the regenerates are evaluated affects the amount of somaclonal variation.

Differences in the concentration of BA may have been responsible for the observed variability between this study and Wakasa's. Wakasa (1979) reported that certain sub-cultures were made on media containing up to 44 uM of BA. Cultivar differences may have also contributed to the variability between these 2 studies. Cultivar differences in somaclonal variability have ben found in sugarcane (Liu and Chen, 1976) and Pelargonium (Skirvin and Janick, 1976). 'Smooth Cayenne' may be very susceptible to both in-vivo and in-vitro induced mutation. Collins (1960) reported that 'Smooth Cayenne' is highly mutable, e.g., the frequency of reversion from smooth leaves to spiny is approximately 1%. The phenetic studies of Chapter VI indicate that the 'Smooth Cayenne' group shows significant isozyme differences from

the other major groups of pineapple cultivars. The isozyme banding patterns of 'Smooth Cayenne' appear to possess more primitive allozymes. One explanation for the observed higher level of somaclonal variation may be that 'Smooth Cayenne' is highly heterozygous and has been vegetatively propagated over a longer period of time than the cultivars examined in this study. This heterozygous condition would then allow for the expression of a greater number of recessive mutants than would be expected in more homozygous cultivars, where a single base pair mutation would not be expressed in the heterozygous condition.

In conclusion, the studies of this Chapter indicate that the clonal fidelity of the tissue culture-regenerated plants has been maintained in the pineapple cultivars tested. These results suggest that tissue culture may be a viable alternative to traditional methods of pineapple propagation. No definite conclusion can be made in regard to the reported 34% somaclonal variation observed in 'Smooth Cayenne' (Wakasa, 1979) relative to the 0.2% observed in this study.

Table 7.1. Regenerated pineapple plants evaluated for electrophoretic and morphological variation.

Cultivar[z]	Electrophoretic		Morphological	
	Regenerated plants (no.)	Zymograms	Regenerated plants (no.)	Variants[y] (no.)
Perolera	189	378	850	2
PR-1-67	201	402	500	3
PR-1-67	310	620	400	1
A. bracteatus var. *tricolor*	67	134	150	0
Total	767	1534	1900	6

[z]Two different plants of 'PR-1-67' were initiated in culture.

[y]Plants showing permanent variant character were included.

A

B

Figure 7.1. Abnormal pineapple regenerated plants. A. Narrow leaves (left) and normal leaves (right); B. Albino striped leaves.

CHAPTER VIII
SUMMARY AND CONCLUSIONS

There is considerable speculation in the literature on the in vitro culture of pineapple and its usefulness as a rapid and efficient propagation method; however, few reports have been published on the number of plants established in soil. Furthermore, most research was conducted with 'Smooth Cayenne' which reportedly has high levels of somaclonal variation in the regenerated plants.

One goal of this research was to develop an efficient in vitro propagation system for pineapple, using different cultivars and species, and to determine the amount of somaclonal variation present in the regenerated plants. High levels of somaclonal variation would significantly reduce the attractiveness of tissue culture as a method for mass propagation. In contrast, high levels of heritable and stable somaclonal variation may be an important source of genetic diversity for pineapple cultivar development and improvement schemes.

A system utilizing liquid shake cultures derived from axillary buds of crown, slips, and stems was devised. Cultures were initiated from excised axillary buds on a

solid MS medium supplemented with 3% sucrose, 0.8% Difco Bactor-agar, 0.57 mM inositol, 1.2 uM thiamine HCl, 10.8 uM NAA, and 8.8 uM BA. Explants were subcultured to fresh medium at 6 week intervals. Proliferating cultures were transferred to liquid medium for multiplication and maintenance. Subculturing was done at 4 week intervals. Transferring to a medium without plant growth regulators to induce root formation was not necessary for plantlet survival. Using this method, approximately 350 plantlets from each bud culture could be obtained in 13 months. No apparent loss in regeneration ability of the cultures has been observed over a 2 year period. Plantlets longer than 3.0 cm had a survival rate of nearly 100% when transferred to soil.

To evaluate the genetic fidelity of the regenerated pineapple plants, morphological observations and protein electrophoresis were employed. Phenotypic variability was observed among the regenerated plants, but the frequency of the variation was significantly lower than in previous reports. Although high levels of somaclonal variation were observed one month after the plants were transferred to soil, plants that were originally variant developed a normal phenotype after new leaves were formed.

A method was devised whereby leaf sap proteins could be assayed for isozyme polymorphism using starch gel electrophoresis. Using this technique, several enzyme systems were identified that proved useful for Ananas

cultivar and species identification, for the evaluation of somaclonal variation, and for use as genetic markers. Consistent and well resolved nonvariable banding patterns were observed for isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphohexose isomerase, superoxide dismutase, and triose phosphate isomerase. Well resolved and variable banding patterns were observed for peroxidase and phosphoglucomutase. Genetic analysis revealed that these 2 enzyme systems are both under the control of 3 loci with 2 alleles each in the crosses examined. These systems are inherited independently and may be used as genetic markers in pineapple. Linkage studies could be carried out between these loci and loci governing morphological traits, especially those of economic importance. If such linkages are discovered, it could significantly reduce the selection generation period, which is 5 years with traditional pineapple breeding methods. In the case of the the Pgm-1 locus, 3 other alleles were present in the genotypes surveyed. Variability was also detected for malate dehydrogenase, but no genetic analysis was possible using the 2 segregating populations available. Until further segregating populations can be analyzed, this enzyme system will probably be of limited use. Improved protein extraction procedures as well as modifications of the existing buffer systems might be required for the successful analysis of these enzyme systems.

The genus Ananas has received a variety of taxonomic treatments since the first pineapples were botanically described. Confusion still exists as to which species should be recognized. Collections of feral types are difficult to classify within the presently delimited species, as they sometimes show characteristics intermediate between species. Another purpose of this study was to electrophoretically characterize the genus Ananas and the pineapple cultivars as an aid to their classification. A taxonomic survey of 67 genotypes was conducted utilizing the peroxidase and phosphoglucomutase staining systems. Fifteen of the 27 pineapple cultivars could be uniquely distinguished. The origin of some cultivars was determined. 'PR-1-67' was confirmed to be a hybrid of 'Red Spanish' x 'Smooth Cayenne'. Similarly, the origin by sporting of 'Esmeralda' and 'Dupuis Smooth' from 'Smooth Cayenne' and 'Cumanesa' from 'Red Spanish' was demonstrated. Five of the 7 Ananas species could be distinguished based on banding patterns of peroxidase and phosphoglucomutase. The 2 exceptions were A. comosus and A. monstrosus. Ananas comosus showed high polymorphism in its cultivars. Ananas monstrosus plants were confirmed to be crown mutations of pineapple cultivars and therefore this should not be considered a valid species. Most of the feral types could be grouped under species with similar morphology and isozyme banding patterns.

Phenetic relationships in Ananas based on isozyme analysis indicate a close relation among the pineapple

cultivars in comparison to the feral types. Evidence of the origin of 'Smooth Cayenne' based on cluster dendogram analysis indicates that it may have been collected in the basin of the Orinoco River and that it still resembles the feral types growing in this region. One of the feral types showed a unique banding pattern that suggested that it may be a distinct taxon.

Electrophoretic evaluation of peroxidase and phosphoglucomutase isozymes from regenerated pineapple plants was done to determine if genetic changes had occurred. All regenerated plants were found to be invariable in their isozyme banding patterns. The presence of some morphologically variant plants among the regenerated plants analyzed suggested that somaclonal variation may be occurring at a low level in these cultivars. However, this variation has not affected the small part of the genome which codes for the enzymes used in the analysis. Further studies should be conducted to determine the extent of the variation occurring in regenerated plants from these genotypes. Particularly important is the need to grow the regenerated plants to maturity and evaluate flower and fruit characteristics before final conclusions on pineapple somaclonal variation are drawn. Similar studies should be undertaken with other cultivars and species to determine whether the genetic background of the pineapple material placed into culture has a definitive relation to the level of phenotypic stability observed. These suggested studies

may generate the information necessary to determine whether uniform or variant plants are produced from pineapple tissue culture.

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BIOGRAPHICAL SKETCH

Maria Grazia Antoni DeWald was born in Rome, Italy, on October 16, 1948. At the age of 3, she moved to Venezuela were she obtained primary and secondary education. She received the degree of Ingeniero Agronomo from the Universidad Central de Venezuela in June, 1975. During the last 2 years as an undergraduate, she was employed as research asistant working on an alfalfa breeding program.

She was appointed after graduation as instructor professor in the Department of Agronomy (Fruit Crops) at the same university and promoted to assistant professor in 1980.

She enrolled as a graduate student in the Fruit Crops Department of the University of Florida in January, 1981, sponsored by the Consejo de Desarrollo Cientifico y Humanistico and the Universidad Central de Venezuela. She received the degree of Master of Science in August, 1983, working in the area of taxonomy and cytogenetics.

In September, 1983, Mrs. DeWald enrolled again in the Fruit Crops Department at the same university as a Ph.D student, this time in the area of plant biotechnology.

The author and her husband, Stephen, are parents of a 2-year-old son, Stephen Francis.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Wayne B. Sherman

Wayne B. Sherman, Chairman
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gloria A. Moore

Gloria A. Moore, Cochairman
Associate Professor of
Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard E. Litz

Richard E. Litz
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul Lyrene

Paul M. Lyrene
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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